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CORRECTIONS

Volume 37, 1959

Page 1318. In the headnote of Table II, (P) after "hypophysectomized" should read (H).

Volume 38, 1960

Page 505. In the paragraph beginning "As soon as they were dry, . . .", the second sentence should read: "They were then sprayed on the other side with diazotized sulphanilic acid (DSA) prepared, at 0° C, by mixing one volume of a solution of sulphanilic acid (0.9 g) in water (1 l.) containing concentrated hydrochloric acid (9 ml) with one volume of aqueous sodium nitrite (0.05%), and 15 minutes later, three volumes of aqueous potassium carbonate (4.0%)".

Page 652. In the last two footnotes to Table I the symbols § and ‖ should be reversed. The footnote designated by § applies only to experiment 4; the one designated by ‖ applies to the 15% oleic acid in experiments 4, 5, and 6.

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THE INFLUENCE OF ADRENALECTOMY AND INDIVIDUAL STEROID HORMONES UPON THE METABOLISM OF ACETATE-1-C^{14} BY RAT LIVER SLICES

I. INCORPORATION INTO PROTEINS[1]

J. S. WILLMER AND T. S. FOSTER

Abstract

The influence of the adrenal gland on incorporation of acetate-1-C^{14} into rat liver protein was studied. Cortisone, corticosterone, and deoxycorticosterone were found to increase isotope incorporation in both sham-operated and adrenalectomized groups, while Reichstein's substance S increased radioactivity in the protein only from sham-operated animals. The effects of the alcohol and acetate forms of cortisone, hydrocortisone, deoxycorticosterone, and Reichstein's substance S were compared.

Introduction

Despite the fact that knowledge and understanding of the influence of adrenocortical hormones on intermediary metabolism has been greatly enlarged by the continuing research in this field, it has not yet been possible to designate with certainty more than a few reactions which are mediated through the cortical hormones.

The effects of the adrenocorticoids upon various metabolic pathways have been studied in a variety of isolated tissues, in intact experimental animals, and in man. It has been established that the cortical secretion influences to a major degree the metabolism of carbohydrate, protein, and fat by the mammalian organism, though the assessment of valid physiological functions is complicated by the many contradictory results obtained.

The present investigations were undertaken to determine the effects of in vivo administration of adrenal steroids upon the metabolism of C^{14}-labelled substrates by rat liver slices. The influence of individual corticoids was measured by utilizing adrenalectomized rats in which replacement therapy with one steroid was maintained during the postoperative period: a comparison was made with the effect of the same steroid upon sham-operated animals. The adrenal steroids investigated were cortisone, hydrocortisone, deoxycorticosterone, Reichstein's substance S, and corticosterone together with their precursor progesterone. In view of the role of the glucocorticoids in gluconeogenesis a study was initially made of the incorporation of isotope from acetate-1-C^{14} into liver protein.

[1]Manuscript received April 1, 1960.
Contribution No. 27, from the Animal Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Ontario.

Methods

Corticosterone, cortisone, deoxycorticosterone, hydrocortisone, Reichstein's substance S (all in the alcohol form), and progesterone, together with the acetates of cortisone, deoxycorticosterone, hydrocortisone, and Reichstein's substance S, were purchased from Mann Research Laboratories Inc., New York 6, New York. In the present investigation a daily hormone dose of 2 mg was used, and it was anticipated that by using a sesame oil/propylene glycol vehicle, release of the steroid would be prolonged, and would approximate physiological levels more closely. However, this does not appear to be true for progesterone as Forbes (1) and Cohen (2) have shown since these experiments were initiated.

Sodium acetate-1-C^{14} was made up to 0.01 *M* with inactive carrier acetate and stored in the deep freeze. The activity of this solution was approximately 450,000 c.p.m./μmole.

Male albino rats of the Wistar strain weighing 70–90 g were used throughout these investigations. Bilateral adrenalectomies or sham operations were performed on the animals under Nembutal anaesthesia by the dorsal approach technique. The rats were maintained in individual cages on fox cubes and drinking water containing 1% sodium chloride. Groups of adrenalectomized or sham-operated animals received one daily intraperitoneal injection of either the vehicle or one of the steroids. This treatment was continued for 7 days postoperatively. Finally, as further controls, two groups of intact rats were used, one of which received no dose, while the other received a daily dose of the vehicle.

On the eighth postoperative day the animals were killed by decapitation, and the completeness of adrenalectomy ensured by gross examination. The liver was quickly removed, placed in ice-cold Krebs–Ringer phosphate buffer solution, and slices prepared using a Stadie-Riggs microtome.

Incorporation of the labelled substrate into protein was studied by incubation of 150 to 200 mg liver slices in 5.0 ml Krebs–Ringer–glucose–phosphate solution with 0.5 ml 0.01 *M* sodium acetate-1-C^{14}. In a comparative study, no differences in incorporation of isotope were observed when either Krebs–Ringer phosphate or bicarbonate media was used. All incubations were carried out at 37.5° C in a Dubnoff bath, in an atmosphere of oxygen.

The protein was isolated by a modification of the method of Kay and Entenman (3) after the incubations had been arrested by the addition of 1.0 ml 10% trichloroacetic acid and 0.5 ml 5 *N* sulphuric acid. The precipitate was washed three times with distilled water by homogenization in an all-glass apparatus. Lipids were removed by two 30-minute extractions at 50° C with 10 ml portions of a 3:1 mixture of ethanol and ether. The residue was washed three times with redistilled acetone and made up to 10.0 ml volume. Aliquots of 2.0 ml were padded and weighed on No. 50 filter paper (4), and 1.0 ml aliquots were similarly padded and counted in an end-window gas-flow counter. After correction for background and self-absorption the data were expressed as c.p.m./mg dry liver.

The average weight changes for all experimental groups of rats are presented

in Table I. The means of duplicate analyses, indicating the degree of substrate incorporation into liver protein obtained from the same animals, are shown in Tables II and III.

Results

It was felt advantageous in this investigation to relate the degree of incorporation of labelled substrate into liver protein to the gain in weight of the experimental animals. The average weight changes for groups treated with the alcohol or acetate forms of the hormones are presented in Table I. No

TABLE I

Average weight changes (g) of experimental animals during an 8-day period

Treatment	Unoperated	Sham-operated	Adrenalectomized
No dose	26.9 (11)		
Solvent	23.3 (12)	25.6 (32)	9.4 (24)
Corticosterone		19.4 (5)	11.5 (5)
Cortisone		19.7 (7)	12.3 (7)
Deoxycorticosterone		21.4 (7)	20.8 (15)
Hydrocortisone		23.0 (7)	9.8 (7)
Progesterone		20.2 (5)	6.8 (7)
Reichstein's substance S		17.3 (6)	13.0 (9)
Cortisone acetate		12.1 (7)	8.7 (7)
Deoxycorticosterone acetate		24.2 (8)	19.0 (6)
Hydrocortisone acetate		3.1 (7)	2.7 (7)
Reichstein's substance S acetate		27.7 (7)	16.5 (6)

NOTE: Values in parentheses are numbers of animals in each group.

statistical evaluation of these data has been attempted, but the more pronounced effects observed are reflected in the degree of isotope incorporation into liver protein. Deoxycorticosterone, both in the alcohol and acetate form, is capable of restoring the growth rate of adrenalectomized rats to almost normal levels. The effects of the alcohol and acetate forms of cortisone and hydrocortisone were observed to be very different, the acetates in each case considerably reducing the gain in weight. The growth rates of the groups receiving Reichstein's substance S acetate were raised.

The influence of the alcohol forms of the hormones on protein synthesis is shown in Table II. A significantly greater incorporation of isotope was observed in adrenalectomized animals than in sham-operated controls. All the hormones studied were capable of restoring protein synthesis in sham-operated animals to the level of the unoperated controls, and a significant increase above this level was observed in animals treated with corticosterone, cortisone, deoxycorticosterone, or Reichstein's substance S. Corticosterone, cortisone, and deoxycorticosterone also increased incorporation in adrenalectomized rats.

As was the case for the growth data, esterification of the hormones (Table III) produces some significant changes. In the cases of cortisone acetate, deoxycorticosterone acetate, and hydrocortisone acetate there is a generally diminished incorporation of isotope into the tissue protein by comparison with the hormone alcohols. Incorporation of isotope by adrenalectomized animals treated with hydrocortisone acetate is significantly lower than in the adrenal-

TABLE II

Effects of adrenal steroid alcohols upon the incorporation of acetate-1-C^{14} into rat liver protein in vitro[a]

Treatment	Unoperated	Sham-operated	Adrenalectomized
No dose	273 ± 36		
Solvent	300 ± 41	218 ± 16	291 ± 16
Corticosterone		609**[b] ± 62	506**[b] ± 41
Cortisone		363*[b] ± 26	494**[b] ± 27
Deoxycorticosterone		412*[b] ± 43	431**[b] ± 24
Hydrocortisone		317[b] ± 20	337 ± 41
Progesterone		357[b] ± 27	262 ± 21
Reichstein's substance S		516**[b] ± 69	312 ± 28

[a]Protein activity expressed as c.p.m./mg dry liver ± S.E.M.
[b]Significant at 1% level as compared with corresponding control group.
*Significant at 5% level as compared with unoperated controls.
**Significant at 1% level as compared with unoperated controls.

TABLE III

Effects of adrenal steroid acetates upon the incorporation of acetate-1-C^{14} into rat liver protein in vitro[a]

Treatment	Unoperated	Sham-operated	Adrenalectomized
No dose	273 ± 36		
Solvent	300 ± 41	218 ± 16	291 ± 16
Cortisone acetate		358*[b] ± 32	404*[b] ± 44
Deoxycorticosterone acetate		404*[b] ± 43	405*[b] ± 41
Hydrocortisone acetate		258 ± 26	221[c] ± 31
Reichstein's substance S acetate		408**[b] ± 30	512**[b] ± 53

[a]Protein activity expressed as c.p.m./mg dry liver ± S.E.M.
[b]Significant at 1% level as compared with corresponding control group.
[c]Significant at 5% level as compared with corresponding control group.
*Significant at 5% level as compared with unoperated controls.
**Significant at 1% level as compared with unoperated controls.

ectomized controls and in the adrenalectomized group treated with hydrocortisone alcohol. Reichstein's substance S acetate causes a pronounced increase in isotope incorporation over the control groups and, in the case of adrenalectomized animals, over the group treated with Reichstein's substance S alcohol.

Discussion

In a comparison of the results obtained in this series of investigations with others of a similar nature, account must be taken of differences likely to govern changes of a physiological nature, by contrast with those that are frankly pharmacological.

The interpretation of results obtained from sham-operated animals is complicated by the stress condition associated with the operation, which is, in itself, a stimulus for increased adrenal secretion. Administration of some corticoids has been shown to cause atrophy of the adrenal gland, with a concomitant decrease in steroid secretion. These circumstances must be considered in conjunction with changes associated with metabolic relationships between the adrenal cortex and medulla. Only in the adrenalectomized animal can the effect of a single hormone be observed and this again does not take into account the possible interdependence of the medulla and cortex.

Bearing these limitations in mind, we made an attempt to relate the above findings to those already published, and to integrate them into an area of research where considerable confusion exists.

As was first demonstrated by Long *et al.* (5) the prime function of the C-11 oxysteroids is probably connected with gluconeogenesis, under which circumstances tissue protein may be catabolized to give rise to glucose. Although the data presented in Table I indicate weight increases in all groups except those treated with hydrocortisone acetate, it would not be possible to judge their nitrogen-balance status on this evidence alone. However, this information, together with the known pronounced effects of certain steroids upon liver transaminase levels (6–8) would indicate that it is possible that protein synthesis is also accelerated under certain conditions.

The elevated incorporation of isotope into protein in adrenalectomized rats in comparison with sham-operated controls confirms the findings of Wool and Weinshelbaum (9), who used rat diaphragm muscle. These workers observed that administration of cortisone to adrenalectomized rats diminished the isotope level in the diaphragm protein. Confirmation of this is found in the observations of Clark (10). In contrast to this, Clark (10) and Yudaev, Lebedeva, and Zavial'skaya (11) using isotopically labelled glycine noted that cortisone caused increased incorporation into liver protein.

Among the glucocorticoids studied above, cortisone and corticosterone have been shown to cause increased hepatic incorporation of isotope in both sham-operated and adrenalectomized animals, whereas hydrocortisone did not show any pronounced effect. This was felt to be surprising in view of the profound influence of this hormone upon transaminase systems (6–8). It is emphasized that in interpreting data of this nature consideration must be given to the relative biological potency of the hormone, the hormonal concentration at the target tissue, and the sensitivity of the individual tissues to particular hormones. The varying potencies of the adrenal steroids in particular physiological functions are well known, hydrocortisone being accepted as the most potent naturally occurring glucocorticoid. While it has not been established, it is suggested that a possible increase in protein turnover in the presence of low glucocorticoid concentrations may be succeeded by an inhibition of protein synthesis as the dose level is increased. Due to their different biological potencies, the glucocorticoids might, under these circumstances, either increase protein synthesis as in the case of cortisone and corticosterone, allow protein synthesis at a normal rate as with hydrocortisone, or depress synthesis at higher dose levels.

The influence of deoxycorticosterone upon incorporation of acetate into liver protein can not be explained by any of its known effects, though it has been found to behave similarly by Yudaev *et al.* (11) using glycine-1-C^{14}. It is possible that the influence of this steroid upon protein synthesis is a secondary manifestation of an altered salt balance, or of an effect upon another endocrine system.

In general the influence of esterification has been to reduce the effect observed with the steroid alcohol, this being particularly noticeable in a study of the

influence of adrenalectomy upon cholesterol synthesis (12). The difference in therapeutic properties of acetate and alcohol forms of the adrenal steroids has been known for some time (13). Segaloff (14) has stated that hydrocortisone acetate may be regarded as a hormone with a prolonged effect, in contrast to the shorter period of activity exhibited by hydrocortisone alcohol. It is perhaps significant that in the groups of rats treated with hydrocortisone acetate, the incorporation of isotope into protein is lower than in the hydrocortisone alcohol-treated groups, possibly indicating a shift in the equilibrium towards protein catabolism.

The difference in effects exerted by the alcohol or acetate forms of the steroids may be partially explained by one or both of two hypotheses. Considerable emphasis has been placed in recent years upon the transport of cortical steroids in the peripheral circulation and upon the nature of the complexes in which they are present (15). An explanation for the general effects of esterification as observed in the above results might possibly lie in a defect in conjugation and hence a reduced effect upon the target tissue or organ. It is also possible that an explanation may be found in the investigations of Wiesel (16), which indicate that the glucogenic corticosteroids, cortisone, and hydrocortisone may produce at least some of these effects by chelating the essential metal portion of intracellular enzyme systems. Wiesel has pointed out that esterification of these hormones with acetate at the C-17 position blocks the active site for metal chelation.

Acknowledgments

The technical assistance of J. M. Fenning is gratefully acknowledged; maintenance and dosing of the animals was carried out by the staff of the Institute's Small Animal Laboratory.

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THE INFLUENCE OF ADRENALECTOMY AND INDIVIDUAL STEROID HORMONES UPON THE METABOLISM OF ACETATE-1-C^{14} BY RAT LIVER SLICES

II. INCORPORATION INTO CHOLESTEROL[1]

J. S. Willmer and T. S. Foster

Abstract

A study has been made of the effect of adrenalectomy upon cholesterol synthesis by rat liver slices. An increased incorporation of acetate-1-C^{14} was observed in adrenalectomized control animals, and in all groups of adrenalectomized rats treated with the steroid hormones under investigation. Deoxycorticosterone was the only hormone used which raised cholesterogenesis from acetate above the level of the adrenalectomized control group: this effect was not observed using mevalonate-2-C^{14} as substrate. In groups of sham-operated animals, deoxycorticosterone, progesterone, and Reichstein's substance S increased acetate incorporation above the level attained by the control group. In a comparison of the effects of certain steroid acetates, Reichstein's substance S acetate and deoxycorticosterone acetate stimulated cholesterogenesis above the levels of the corresponding controls in both sham-operated and adrenalectomized animals, though the effect of the latter hormone was not as pronounced as when the alcohol form was used.

Introduction

The mechanism of cholesterogenesis is of considerable interest and has been greatly clarified in recent years. The role of certain hormones in regulating this process has been widely investigated. Liver slices from diabetic animals (1) have a greater capacity for cholesterol synthesis from acetate than slices from normal animals. Thyroxine-treated rat livers have also been observed (2) to produce more cholesterol under similar in vitro conditions. The influence of adrenalectomy, as demonstrated by Perry and Bowen (3), was to depress the capacity of liver slices to incorporate radioactive acetate into cholesterol and fatty acids. In view of the considerable evidence indicating the participation of the adrenal cortex in carbohydrate, protein, and lipid metabolism, a further investigation into the influence of adrenalectomy and individual steroid hormones upon the system was initiated. A comparison was made between cholesterogenesis in liver slices from adrenalectomized, sham-operated, and intact animals, and the effects of daily doses of cortisone, hydrocortisone, deoxycorticosterone, corticosterone, Reichstein's substance S, or progesterone upon these groups of animals were observed.

Methods

The treatment and maintenance of animals was as described previously (4), all hormones being administered intraperitoneally as daily doses of 2 mg for 7 days.

Tissue slices 0.5 mm in thickness and weighing 150–200 mg, were incubated in an atmosphere of 100% oxygen, with 5.0 ml Krebs–Ringer–glucose–phos-

[1]Manuscript received April 4, 1960.
Contribution No. 28 from the Animal Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Ontario.

phate buffer solution and 0.5 ml 0.01 *M* sodium acetate-1-C^{14} containing approximately 2,250,000 c.p.m. Incubation was for 2 hours at 37.5° C.

Cholesterol was isolated and purified after saponification of the tissue by the method of Migicovsky (5). The purified cholesterol was precipitated as the digitonide, padded according to the method of Migicovsky and Evans (6), and counted in an end-window gas-flow counter. After the cholesterol digitonide was weighed directly, activity was corrected for background and self-absorption, and expressed as c.p.m./mg dry liver. All results are presented as the mean of duplicate analyses from groups of at least five animals ± the standard error.

Results

The incorporation of acetate-1-C^{14} into cholesterol by rat liver slices was significantly increased in the adrenalectomized controls as compared with the sham-operated or intact controls (Table I). Treatment with deoxycorticosterone resulted in a further significant increase in adrenalectomized rats.

TABLE I

Effects of adrenalectomy and adrenal steroid alcohols upon incorporation of acetate-1-C^{14} into cholesterol by rat liver slices[a]

Treatment	Unoperated	Sham-operated	Adrenalectomized
No dose	217 ± 32		
Solvent	249 ± 64	217 ± 14	334** ± 22
Corticosterone		228 ± 16	308* ± 80
Cortisone		221 ± 36	303* ± 40
Deoxycorticosterone		364**[b] ± 41	1219**[b] ± 70
Hydrocortisone		224 ± 45	416** ± 35
Progesterone		421**[b] ± 44	338** ± 34
Reichstein's substance S		433**[b] ± 71	371** ± 32

[a]Cholesterol activity expressed as c.p.m./mg dry liver ± S.E.M.
[b]Significant at 1% level as compared with corresponding control group.
*Significant at 5% level as compared with unoperated controls.
**Significant at 1% level as compared with unoperated controls.

Incorporation in adrenalectomized groups treated with the remaining steroids was not significantly different from that in the adrenalectomized controls, but was in all cases higher than in the intact controls. In groups of sham-operated animals which received progesterone, deoxycorticosterone, or Reichstein's substance S, the isotope content was significantly increased over that in the sham-operated or intact control groups.

In view of the general difference in effects exerted by the steroid acetates upon the incorporation of radioactive acetate into liver protein (4), the influence of these compounds upon cholesterogenesis was investigated. The same general effect was observed (Table II), again with certain outstanding anomalies, the influence of esterification being to cause a lower isotope incorporation than was observed with the steroid alcohols. Whereas the groups which were treated with the acetates of cortisone or hydrocortisone showed no increase in isotope incorporation above the sham-operated or intact control levels, those which received the acetates of deoxycorticosterone or Reichstein's substance S exhibited a significantly increased activity. There was, however, a

TABLE II

Effects of adrenalectomy and adrenal steroid acetates upon incorporation of acetate-1-C^{14} into cholesterol by rat liver slices[a]

Treatment	Unoperated	Sham-operated	Adrenalectomized
No dose	217 ± 32		
Solvent	249 ± 64	217 ± 14	334** ± 22
Cortisone acetate		148 ± 23	273 ± 46
Deoxycorticosterone acetate		423**[b] ± 66	436**[c] ± 50
Hydrocortisone acetate		156 ± 74	277 ± 68
Reichstein's substance S acetate		311**[b] ± 46	446**[c] ± 55

[a]Cholesterol activity expressed as c.p.m./mg dry liver ± S.E.M.
[b]Significant at 1% level as compared with corresponding control group.
[c]Significant at 5% level as compared with corresponding control group.
**Significant at 1% level as compared with unoperated controls.

pronounced difference between the effects of deoxycorticosterone and deoxycorticosterone acetate, the former increasing cholesterogenesis from acetate sixfold, while the latter only doubled incorporation.

In order to ascertain whether deoxycorticosterone exerted a similar effect at some further stage in the biosynthesis of cholesterol, mevalonic acid-2-C^{14} was used as the substrate in a similar series of investigations. While neither deoxycorticosterone nor any of the other steroids stimulated cholesterogenesis, isotope incorporation was again higher in the adrenalectomized control group than in the sham-operated group. This increased activity in adrenalectomized animals as compared with their corresponding sham-operated group was also observed in those treated with deoxycorticosterone, hydrocortisone, progesterone, or Reichstein's substance S, though the latter steroid caused a depression in cholesterogenesis in both groups. Differences were, however, in no case as great as were observed with acetate-1-C^{14} as substrate.

Discussion

It has been shown in this investigation that the effect of adrenalectomy is to increase the ability of the liver to synthesize cholesterol. A possible explanation for this lies in the findings of Fregly (7), and of Fletcher and Myant (2). Fregly, during investigations into the production of hypertension in adrenalectomized rats by hypertonic salt solutions, observed that whereas no hypertension was produced by 0.9% saline in either control or adrenalectomized animals, the latter group of animals, maintained on 0.9% saline, exhibited a significant increase in thyroid weight. Fletcher and Myant showed that a two- to three-fold increase in hepatic cholesterogenesis from acetate could be obtained by treating rats with thyroxine. It would seem possible that the increased cholesterol synthesis observed in adrenalectomized rats may be an indirect effect due to an elevated basal metabolic rate mediated through the thyroid gland. The effect of adrenalectomy in stimulating cholesterol biogenesis is at variance with the findings of Perry and Bowen (3). They reported that incorporation of acetate-2-C^{14} into fatty acids and cholesterol, by liver slices of intact and adrenalectomized rats, was depressed in the latter group. It is important in comparing these results to relate them to the physiological condition of the experimental animals. Migicovsky (unpublished observations)

has demonstrated that cholesterol biogenesis in vitro is reduced in older rats, and it is possible that this might be the source of the different results.

The predominant finding in this series of investigations is the pronounced effect of deoxycorticosterone in stimulating cholesterogenesis from acetate. This influence is quite distinct from any exerted by the other steroids studied. Since no increase on a comparable scale was observed with labelled mevalonic acid as substrate, it seems possible that this adrenocorticoid activates in some way an enzyme or enzymes involved in cholesterol synthesis prior to mevalonic acid. That this is a relatively specific effect of deoxycorticosterone on lipid metabolism is reflected in the fact that while it increased acetate incorporation into liver protein (4), and also trebled acetate oxidation (8), a sixfold increase in cholesterol activity was observed. A possible explanation for the effect of this hormone may be found in its known hypertensive action, which is particularly evident when accompanied by an increased intake of sodium chloride (9). Omae and Masson (10) have shown that cortisone and hydrocortisone also are capable of producing hypertension in rats, but have suggested that the mechanisms of mineralocorticoid and glucocorticoid hypertensions are different. Rats treated with deoxycorticosterone acetate maintained an elevated blood pressure after discontinuation of treatment; this was not found to occur with the two glucocorticoids.

The over-all picture of the effects of adrenal steroids upon lipid metabolism is very confused, many species variations being observed. The administration of cortisone or hydrocortisone to rabbits was shown by Wang, Bossak, and Adlersberg (11) to produce increased serum concentrations of phospholipid, neutral fat, and cholesterol. A similar effect of cortisone in adrenalectomized dogs could be overcome by simultaneous treatment with deoxycorticosterone acetate (12). Knowlton *et al.* (13) and Migeon (14) were unable to show any significant changes in serum cholesterol or lipid phosphorus levels in adrenalectomized rats treated with deoxycorticosterone acetate. However, as has been demonstrated in the case of the thyroid gland the effect of a particular hormone upon the plasma cholesterol level is not necessarily reflected in the rate of hepatic cholesterogenesis (15). Whether the effect of deoxycorticosterone upon cholesterol synthesis in the liver is exerted directly or indirectly is a matter of conjecture, but it would appear possible, in view of the known principal function of this steroid as a mineralocorticoid, that some other endocrine system is implicated.

It has been reported by Hotta and Chaikoff (1) that cholesterogenesis is also increased in the diabetic animal, though this has not been confirmed by the extensive studies of Siperstein and Fagan (16). The importance of the hexose-monophosphate shunt in the synthesis of cholesterol and in lipogenesis has been emphasized by these latter investigators, and it is perhaps pertinent to note that the level of glucose-6-phosphate dehydrogenase is elevated in livers of thyroxine-treated rats (17) and in adrenalectomized rats (18), but decreased in alloxan-diabetic animals (17). Work in this laboratory (19) has confirmed that the glucose-6-phosphate dehydrogenase activity of rat liver is raised above the control level 3 days postoperatively, though deoxycorticosterone was not found

to influence the activity of this system.

If it is to be accepted that diabetic animals do indeed have a higher capacity for cholesterol synthesis it is surprising that neither cortisone nor hydrocortisone, which may cause "steroid diabetes", produce an increase in hepatic cholesterogenesis in adrenalectomized or sham-operated animals.

Acknowledgments

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THE INFLUENCE OF ADRENALECTOMY AND INDIVIDUAL STEROID HORMONES UPON THE METABOLISM OF ACETATE-1-C^{14} BY RAT LIVER SLICES

III. RESPIRATORY EFFECTS[1]

J. S. WILLMER AND T. S. FOSTER

Abstract

The oxidation of acetate-1-C^{14} by rat liver slices was found to be raised above the control level in both sham-operated and adrenalectomized animals. Deoxycorticosterone further increased substrate oxidation in the adrenalectomized group, but had no effect upon sham-operated animals. No other hormone investigated influenced the oxidation of acetate. The respiratory rate of liver slices was elevated by adrenalectomy: cortisone and deoxycorticosterone lowered the respiratory rate of sham-operated animals. A comparison has been made with the effects of certain esterified steroids. These were, in general, observed to be less stimulatory in effect than the corresponding alcohols, and in some cases caused considerable inhibition of respiration.

Introduction

Changes in respiratory rates, both of intact animals and isolated tissues, are frequently used as an indication of a modified metabolism. The influence of many factors, including hormones, upon respiration has been studied.

Evidence for respiratory changes induced by adrenal hormones is voluminous, though confused, particularly as no standard can be set for experimental conditions, and the effects recorded are as varied as the particular tissues and hormones involved. While it has been observed that adrenalectomized rats produced a significantly greater amount of $C^{14}O_2$ from injected radioactive pyruvate than did intact controls (1), Perry and Bowen (2) reported no significant change in acetate oxidation by liver slices from adrenalectomized rats. Observations concerning the influence of adrenal hormones or adrenalectomy upon the oxidation of glucose (3), fructose (4), or galactose (5) have also been reported but provide little clarification of the mode of action of these hormones.

A similar lack of agreement is present in the available evidence for the influence of these hormones upon the rate of oxygen absorption of isolated tissues. Among those studied have been brain and muscle (6–8), liver (9), kidney (10), skin (11), adipose tissue (12), diaphragm (13), and heart (13).

In the present investigation an attempt has been made to elucidate the influence of individual steroid hormones upon the oxidation of acetate-1-C^{14} by liver slices from adrenalectomized, sham-operated, and intact rats.

Methods

The treatment and maintenance of experimental animals was as previously described (14). The steroids investigated were cortisone, hydrocortisone, deoxycorticosterone, corticosterone, Reichstein's substance S, and progesterone, each being administered as a daily dose of 2 mg for 7 days.

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Contribution No. 30 from the Animal Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Ontario.

Respiration studies were carried out on liver slices weighing 50–70 mg in Warburg flasks containing 2.0 ml Krebs–Ringer–phosphate–glucose medium. The side arms of the flasks contained 0.1 ml of 0.01 *M* sodium acetate-1-C^{14} having an activity of approximately 450,000 c.p.m. Filter papers on which was absorbed 0.2 ml 20% potassium hydroxide were placed in the center wells. All results are expressed in terms of Q_{O_2} (μl O_2 absorbed/hour/mg dry tissue).

Radioactive carbon dioxide was determined by transferring the contents of the center well of the Warburg flask into 10 ml distilled water, adding 1.0 ml 0.4% sodium carbonate to give a suitable pad weight, and precipitating barium carbonate with 1.0 ml 5% barium chloride. The precipitate was padded on filter paper disks (15) and counted in an end-window gas-flow counter. The weight of the pad was determined by dissolving the precipitate in excess 0.1 *N* HCl and back-titrating with alkali. Activity was corrected for background and self-absorption and expressed as c.p.m./mg dry liver.

All results are presented as the mean of duplicate analyses from groups of at least five animals.

Results

The oxidation of acetate-1-C^{14} to $C^{14}O_2$ (Table I) was significantly increased in the sham-operated and adrenalectomized control animals, by comparison with the intact controls, although there was no significant difference in this

TABLE I

The influence of adrenal steroid alcohols on the oxidation of acetate-1-C^{14} by rat liver slices[a]

Treatment	Unoperated	Sham-operated	Adrenalectomized
No dose	1736 ± 102		
Solvent	1697 ± 164	2285** ± 83	2430** ± 140
Corticosterone		2844**[b] ± 299	2720** ± 326
Cortisone		2672** ± 318	3152**[b] ± 337
Deoxycorticosterone		2423** ± 164	6462**[c] ± 351
Hydrocortisone		2192 ± 242	3471**[c] ± 271
Progesterone		2252** ± 145	2438** ± 225
Reichstein's substance S		2486** ± 239	2616** ± 138

[a]$C^{14}O_2$ activity expressed as c.p.m./mg dry liver ± S.E.M.
[b]Significant at 5% level as compared with corresponding control group.
[c]Significant at 1% level as compared with corresponding control group.
**Significant at 1% level as compared with unoperated controls.

capacity between the two former groups. A further significant increase was caused by treatment of adrenalectomized rats with cortisone, deoxycorticosterone, or hydrocortisone, and of sham-operated animals with corticosterone.

These values may be compared with the levels of oxygen uptake obtained from the same animals, presented in Table II. Again adrenalectomy produced a significant increase, though no difference was observed between sham-operated and intact control animals. The values for the adrenalectomized groups which were treated with corticosterone, Reichstein's substance S, or progesterone are not significantly different from those of the intact controls. While the values obtained for the remaining adrenalectomized groups are higher than those for

TABLE II

The influence of adrenal steroid alcohols on the respiration of rat liver slices[a]

Treatment	Unoperated	Sham-operated	Adrenalectomized
No dose	5.6±0.3		
Solvent	5.4±0.3	5.6±0.2	7.1**±0.2
Corticosterone		5.7±0.3	5.6[b]±0.2
Cortisone		4.7*±0.4	6.9*±0.8
Deoxycorticosterone		4.1**±0.3	7.2**±0.3
Hydrocortisone		5.6±0.5	6.5*±0.3
Progesterone		4.8±0.3	6.3±0.3
Reichstein's substance S		6.0±0.8	6.1[c]±0.4

[a]Q_{O_2} expressed as μl O_2 absorbed/hour/mg dry liver ± S.E.M.
[b]Significant at 1% level as compared with corresponding control group.
[c]Significant at 5% level as compared with corresponding control group.
*Significant at 5% level as compared with unoperated controls.
**Significant at 1% level as compared with unoperated controls.

the intact control animals no significant differences from the adrenalectomized controls are evident. Treatment with cortisone or deoxycorticosterone was observed to depress the respiration of liver slices from sham-operated animals.

As in previous publications (14, 16) the influence of the steroid alcohols was in certain cases compared with the effects of administering the hormones as acetates. Again the influence of esterification, both on $C^{14}O_2$ production (Table III) and Q_{O_2} (Table IV), was to cause a diminution in effect. While

TABLE III

The influence of adrenal steroid acetates on the oxidation of acetate-1-C^{14} by rat liver slices[a]

Treatment	Unoperated	Sham-operated	Adrenalectomized
No dose	1736±102		
Solvent	1697±164	2285**±83	2430**±140
Cortisone acetate		2408*±117	2554**±31
Deoxycorticosterone acetate		2219±288	2231*±147
Hydrocortisone acetate		2087±276	2660**±197
Reichstein's substance S acetate		2429**±242	2219*±144

[a]$C^{14}O_2$ activity expressed as c.p.m./mg dry liver ± S.E.M.
*Significant at 5% level as compared with unoperated controls.
**Significant at 1% level as compared with unoperated controls.

TABLE IV

The influence of adrenal steroid acetates on the respiration of rat liver slices[a]

Treatment	Unoperated	Sham-operated	Adrenalectomized
No dose	5.6±0.3		
Solvent	5.4±0.3	5.6±0.2	7.1** ±0.2
Cortisone acetate		5.0±0.3	5.2[b] ±0.2
Deoxycorticosterone acetate		3.9**±0.5	4.6**[b] ±0.4
Hydrocortisone acetate		4.2**±0.4	5.1[b] ±0.3
Reichstein's substance S acetate		5.0±0.4	4.7*[b]±0.3

[a]Q_{O_2} expressed as μl O_2 absorbed/hour/mg dry liver ± S.E.M.
[b]Significant at 1% level as compared with corresponding control group.
*Significant at 5% level as compared with unoperated controls.
**Significant at 1% level as compared with unoperated controls.

deoxycorticosterone caused a threefold elevation in $C^{14}O_2$ production in adrenalectomized animals, administration of deoxycorticosterone acetate caused no

greater change than did adrenalectomy alone. To a lesser extent this was also observed in the groups of animals treated with cortisone acetate or hydrocortisone acetate.

Sham-operated animals treated with deoxycorticosterone acetate or hydrocortisone acetate exhibited a significantly decreased respiratory rate and this is reflected in the reduced activity of CO_2 produced, the values obtained not being significantly different from those of the intact controls.

The steroid acetates reduced the respiratory rate in all groups of adrenalectomized animals to a level which was significantly lower than that of the adrenalectomized control group (Table IV).

Discussion

In an appraisal of the data presented above, account must be taken of the fact that the media in which the tissue slices were incubated contained both acetate and glucose as substrates. While the measurement of $C^{14}O_2$ evolution gives an indication of acetate oxidation alone, the Q_{O_2} values are a function of the oxidation of both glucose and acetate. A comparison of the results in Tables I and II shows that while deoxycorticosterone caused a considerable increase in $C^{14}O_2$ production, it did not stimulate respiration above the adrenalectomized control level. This apparently specific influence upon the metabolism of acetate is confirmed by the stimulation of cholesterogenesis evoked by deoxycorticosterone (16). It is also evident that while either adrenalectomy or the stress imposed by sham operation raised the production of $C^{14}O_2$, no steroids other than deoxycorticosterone acted as a further stimulus to acetate oxidation. This may be compared with the influence upon respiration, which is increased only in adrenalectomized control animals and maintained at this elevated rate by cortisone, deoxycorticosterone, or hydrocortisone: sham operation alone produced no change in respiratory rate, and the imposition of treatments with cortisone or deoxycorticosterone actually caused a reduction in Q_{O_2}.

A general conclusion to be drawn from these data is, therefore, that the effect of adrenalectomy is to stimulate non-specifically the oxidation of both acetate and glucose, and that this effect may be modified by treatment with individual steroids. Deoxycorticosterone apparently increases lipogenesis from acetate; corticosterone, progesterone, and Reichstein's substance S depress respiration in adrenalectomized rats, but exert no influence upon acetate oxidation.

Considerable conflicting evidence already exists for the effects of adrenalectomy and adrenal hormones upon the respiration of tissue slices and intact animals. Contrary to our results, Perry and Bowen (2) reported that adrenalectomy caused no significant change in the oxidation of acetate-2-C^{14} by rat liver slices, though the difference in age and physiological condition of the two experimental groups could account for the dissimilar observations. Confirmation of the evidence presented here may be found in the work of Winternitz and Kline (1), who observed that adrenalectomized rats exhaled significantly greater amounts of $C^{14}O_2$ than normal unoperated controls when they were injected with pyruvate-2-C^{14}. Few results obtained under similar conditions are available for comparison, though the evidence presented in Table II indicates that

adrenalectomy does cause an increase in glucose oxidation. This differs from the findings of Ashmore *et al.* (4), who, using glucose-1-C^{14} and fructose-U-C^{14} as substrates, observed no difference in $C^{14}O_2$ production either in adrenalectomized animals or in hydrocortisone-treated intact rats. In contrast to this it was observed by Tipton (9) that adrenalectomy decreased the respiration of liver slices utilizing pyruvate or succinate, but not with glucose as substrate. This is supported by Russell and Wilhelmi (17), who, using rat kidney slices, found that respiration could be restored to normal or increased by adrenal cortical extract or deoxycorticosterone.

In conclusion we would again emphasize the important part played by the physiological state of the animals; until hormones can be so administered as to allow of utilization at normal physiological levels, conditions must be strictly comparable if the data are to be interpreted correctly.

Acknowledgments

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PURIFICATION OF PROTHROMBIN AND THROMBIN BY CHROMATOGRAPHY ON CELLULOSE[1]

WALTER H. SEEGERS AND RICARDO H. LANDABURU

Abstract

A method is described for obtaining purified bovine thrombin by chromatography on phosphate cellulose, and purified bovine prothrombin by chromatography on diethylaminoethyl cellulose. Both proteins are obtained as single components, and in high yield. The purified prothrombin does not convert to thrombin in 25% sodium citrate solution or protamine sulphate solution. The N-terminal amino acid is the same as in purified prothrombin obtained by different methods in this laboratory, but instead of tyrosine and glycine the C-terminal amino acid is serine. A fundamental difference in structure is thus associated with altered activation characteristics.

In this laboratory methods for the purification of bovine prothrombin were developed and employed to provide material for extensive studies. In principle the prothrombin is precipitated from diluted plasma, adsorbed on magnesium hydroxide, eluted, fractionated with ammonium sulphate, and precipitated at the isoelectric point (1). Thrombin suitable for physical chemical studies was prepared from these products (2) and also concentrates of autoprothrombin I, autoprothrombin II, as well as the recently discovered inhibitor autoprothrombin II-A (3). A simple molecular entity, prothrombin, is thus the source of several natural substances of importance in physiology (4). In addition, acetylated prothrombin (5) and acetylated thrombin (6) were created as modified proteins with properties of great interest (7). To obtain and extend such information we are quite dependent upon the convenience of the procedures because the labor to obtain material is quite extensive in proportion to our requirements. Moreover, modifications and new procedures could conceivably remove important trace substances, increase the specific activity, or even alter the structure of the prothrombin itself.

The introduction of cellulose materials (8) for chromatography of proteins was followed by their commercial production, and in this paper we report our successful experiments for the isolation of prothrombin with a specific activity no higher than previously obtained, but with a structure that is different from any previously described. The method for obtaining this is relatively easy to carry through. With respect to thrombin its production with cellulose is by far the simplest in our experience, but most likely the product is not different from others.

Materials and Methods

Bovine prothrombin was separated by adsorption on magnesium hydroxide (1). The specific activity of the products used ranged from 15,000 to 30,000

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units/mg tyrosine when assayed by the modified two-stage methods (9). Thrombin activity was measured with the use of fibrinogen as a substrate (10). Phosphate cellulose (Cellex-P) was obtained from California Corporation for Biochemical Research, Los Angeles 63, California, U.S.A.; and cellulose coupled with 2-chlorotriethylamine hydrochloride (DEAE cellulose) was obtained from Brown Company, Berlin, New Hampshire, U.S.A.

Chromatography of Prothrombin with DEAE Cellulose

The buffers used in this work are the following:

Buffer A
0.3 *M* pH 8.0
2.194 g $NaH_2PO_4 \cdot H_2O$
40.333 g Na_2HPO_4

Buffer B
0.05 *M* pH 7.0
2.691 g $NaH_2PO_4 \cdot H_2O$
4.331 g Na_2HPO_4

Buffer C
Mix 1 part buffer A
9 parts buffer B
(0.075 *M* pH 7.2)

Buffer D
Mix 5 parts buffer A
5 parts buffer B
(0.175 *M* pH 7.45)

The DEAE cellulose anion exchanger was conditioned by washing several times with phosphate buffer B to obtain the buffer concentration and pH wanted for the chromatography. The small amount of nonsedimenting material was removed by decantation and discarded. The glass tube for the column was 30 cm long and 9 mm in diameter and was brought to a taper at the bottom and closed with filter paper. The cellulose was then mixed a few minutes in a Waring blender, so that the adsorbant could be poured as a slurry, by repeated additions until there was no more settling by gravity. Air pressure (10 p.s.i.) was then applied to obtain a column of cellulose 25 cm long and 9 mm in diameter. When prepared in this way the column does not run dry under gravity.

The prothrombin was first prepared by the magnesium hydroxide adsorption method of Seegers (1). About 100,000 units were taken up in 4 to 5 ml of the 0.05 *M*, pH 7.0, phosphate buffer B and applied to the top of the column. In about 10 to 15 minutes the prothrombin solution usually goes in and can be followed with the phosphate buffer at a rate of about 10 ml per 30 minutes (one drop every 18–20 seconds). About 40 ml of buffer are run through (collect in four tubes). Very little prothrombin comes from the column with this buffer or with the change to the next buffer C (Fig. 1). The 5 ml of the original buffer above the cellulose are not removed, and 70–100 ml of buffer C are run through with no change in rate (one drop every 18–20 seconds). Then buffer D, which contains more salt and is more alkaline, is used to elute the prothrombin. The change is made as described for buffer C. Generally prothrombin can be collected after 30–40 ml of buffer D have been used, and the material with highest potency is in the fourth or fifth tube (Fig. 1).

In a series of five successive preparations the specific activity, expressed in units per milligram tyrosine, in the highest tube was respectively: 33,200, 31,300, 36,300, 39,800, and 31,800. The recovery in all tubes ranged from 82 to

100% of the total applied to the column. We found that the prothrombin should have at least a specific activity of 10,000 or preferably 15,000 units per mg tyrosine before being applied to the column. The recovery was a little better and the specific activity a little higher when the work was done at icebox temperatures rather than in the laboratory.

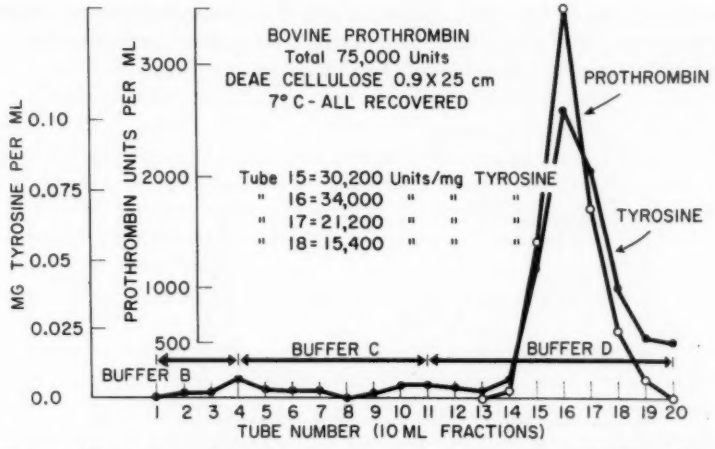

FIG. 1. Chromatography of bovine prothrombin preparation on DEAE cellulose.

Chromatography of Thrombin with Phosphate Cellulose

There are several cellulose preparations available for use in ion exchange chromatography, but so far we have used only phosphate cellulose with success. Diethylaminoethyl cellulose (anionic exchanger), used for our prothrombin work, apparently does not adsorb the thrombin. Carboxymethyl cellulose (cationic exchanger) adsorbs the thrombin, but in the process of chromatography thrombin activity is lost.

For the chromatography with phosphate cellulose the reagents needed are as follows:

Buffer A
0.01 *M* pH 7.0
0.538 g $NaH_2PO_4 \cdot H_2O$
0.866 g $NaHPO_4$

Buffer B
0.05 *M* pH 7.0
2.691 g $NaH_2PO_4 \cdot H_2O$
4.331 g Na_2HPO_4

Buffer C
0.15 *M* pH 8.0
1.097 g $NaH_2PO_4 \cdot H_2O$
20.166 g Na_2HPO_4

The particular phosphate cellulose used contained 1 milliequivalent of phosphorus per g, and was equilibrated at pH 7.0 with buffer A. Usually three or four washings were sufficient. A column 2×13 cm was poured and packed

under gravity. The column was not allowed to run dry. Then about 150,000 units of bovine thrombin, which already had at least a specific activity of 12,000 units per mg of tyrosine, was layered over the column. This thrombin can easily be obtained from prothrombin by one of several activation procedures (11, 12). The thrombin was contained in usually not more than 3 ml of solution. The thrombin adsorbs. Buffer A (one drop every 7 to 10 seconds) was used until 80 to 100 ml of buffer passed. This brought out impurities and very little thrombin (Fig. 2). Then buffer B was used to wash away more nonthrombin protein. About 60 ml were used.

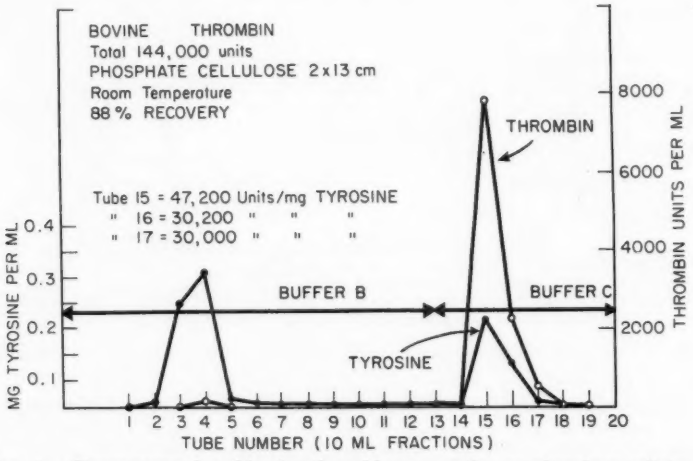

FIG. 2. Chromatography of bovine thrombin preparation on phosphate cellulose.

Finally buffer C was used to chromatograph the thrombin. After about 60 to 80 ml were collected the thrombin appeared rather sharply in a few tubes (10 ml each) and had a specific activity in the range of 47,000 units per mg tyrosine. The buffer C was run at a rate of about one drop every 8 to 10 seconds. This procedure was performed at room temperature. In three successive runs the highest specific activity in terms of units per mg tyrosine was 47,200, 46,400, 41,600. This purification was possible even when the specific activity of the starting material was as low as 10,000. All of the thrombin was recovered, and concentrated by the methods of Seegers, Levine, and Shepard (2).

Discussion

Our method for obtaining thrombin is by far the simplest described to date. Conditioning of the cellulose is usually achieved with a few washings, and the entire chromatography can be completed in 3 hours. After use it can be cleaned with 0.1 *N* sodium hydroxide and used again. The specific activity of the thrombin is the same (2) as for the material obtained on IRC-50(XE-64). The addition of Rivanol to the IRC-50(XE-64) has yielded a product reported to have a specific activity of 61,000 units/mg tyrosine (13). This is a value we

have not been able to achieve and we suppose that differences in the methods of assay account for the unusual observation.

In the chromatography of prothrombin with cellulose, simplicity, reproducibility, and convenience are the outstanding characteristics of the method. The product is homogeneous when examined with an ultracentrifuge. It has a sedimentation constant comparable to that reported previously (14) for prothrombin preparations made in this laboratory. The specific activity is equal to the best obtained by the magnesium hydroxide technic (1) or this technic combined with chromatography on IRC-50(XE-64) as described by Miller (15). Whereas these two last-mentioned procedures give products that transform to thrombin in 25% sodium citrate solution or in protamine sulphate solution, our DEAE-prothrombin does not. This prothrombin preparation thus has about the same specific activity and the same sedimentation constant, but is nevertheless different in activation characteristics. On further study it was found that the N-terminal amino acid is alanine, quite as in the other two products (16). However, the C-terminal amino acid is serine, whereas the other two preparations have tyrosine and glycine (16). There is thus a difference in chemical structure as the C-terminal end(s) of the molecule.

The magnesium hydroxide method of Seegers has been modified with the use of barium sulphate in place of magnesium hydroxide and used to process plasma filtered through a Seitz filter (17). This prothrombin did not form thrombin in 25% sodium citrate solution, and we have already mentioned that this might be due to an inhibitor that can adsorb on barium sulphate (18), or that Seitz filtration alters the plasma components (19). We can now add the further suggestion that the filtration specifically alters the prothrombin (16).

Other alterations of prothrombin seem to be likely, and we cite one more example. Prothrombin made by the method of Seegers (1) can be passed through the amberlite IRC-50(XE-64) column to produce a prothrombin that becomes autoprothrombin I in the presence of thrombin (3, 4). Before the chromatography the same prothrombin becomes autoprothrombin II in the presence of thrombin. This change in activation characteristics is independent of qualitative N-terminal or C-terminal amino acid composition (16). Furthermore, the selected best preparations obtained by the method of Seegers (1) are not made more potent by the IRC-50 chromatography.

In our extensive experience with IRC-50 chromatography as described by Miller (15) we find that the potency of the chromatographed prothrombin is quite dependent upon the specific activity of the starting material. If the specific activity is low after the chromatography the preparations become autoprothrombin I in the presence of thrombin (3). Whether the prothrombin converts to autoprothrombin I or autoprothrombin II depends upon whether it has been in contact with IRC-50 or not, and seems to be independent of specific activity or the N- or C-terminal amino acids.

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CARDIAC OUTPUT IN ACUTE EXPERIMENTAL METHEMOGLOBINEMIA[1]

C. W. GOWDEY

Abstract

Methemoglobinemia induced in normal anesthetized dogs by intravenous infusions of aniline resulted in a decreased arterial oxygen content and a marked increase in cardiac output. Heart rate, arterial pressure, blood viscosity, and oxygen consumption increased, while total peripheral resistance and arteriovenous oxygen difference decreased. The elevation of cardiac output occurred in spite of the fact that the hematocrit level and blood viscosity increased. Ganglion-blocking doses of pentolinium bitartrate did not significantly alter the cardiovascular responses to the methemoglobinemia.

Introduction

During our investigations of the responses of the heart to acute experimental anemia in dogs the possibility arose that a good part of the increased cardiac output might be related to the reduced viscosity of the anemic blood. In 1959 Richardson and Guyton (1) stated that it had not been determined whether the changes in cardiac output during anemia or polycythemia were caused by changes in blood viscosity or by other factors; they concluded that viscosity alone may have a major effect. Much earlier, Whittaker and Winton (2) had demonstrated in the perfused hind limb of the dog that the apparent viscosity of blood decreased as the corpuscular concentration decreased. The relation between viscosity and erythrocyte concentration has been further elucidated by the work of Levy and Share (3) and recently by Haynes and Burton (4).

It was considered that the relative importance of the viscosity factor might be evaluated if the cardiac output were to be measured in experiments in which the capacity of the blood for carrying oxygen was reduced without a concomitant reduction in the hematocrit level. The acute formation of methemoglobin would produce just such conditions, and Clark and his associates (5, 6) had found that the administration of aniline to dogs produced marked methemoglobinemia. Accordingly, the cardiac output was measured in a series of dogs before and at intervals after the reduction of the arterial oxygen content by aniline-induced methemoglobinemia.

Methods

Mongrel dogs weighing between 13.5 and 22.5 kg, average 18.3 kg, were anesthetized lightly with sodium pentobarbital. Catheters were placed in the brachial or femoral artery and the pulmonary artery or right ventricle, and the pressures were measured by Sanborn manometers recording on a Polyviso. All pressure readings were measured from heart level. The cardiac output was determined by the direct Fick method; oxygen consumption was measured

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with a Sanborn Metabulator. The samples of arterial and mixed venous blood were withdrawn over 2-minute periods after the animal had been breathing oxygen for 5 minutes and the blood gases were determined immediately after withdrawal by the method of Peters and Van Slyke. Arterial hematocrit levels were measured in Wintrobe tubes. Total peripheral resistance was calculated as the mean arterial pressure in mm Hg divided by the cardiac output in ml per minute per kg. A period of 2.5 hours from the onset of anesthesia was allowed for the animal to reach a steady state. In the control period which followed, two determinations of the cardiac output and other cardiovascular indices were made. Then aniline (150 mg per kg dissolved in 0.9% saline) was administered intravenously over a 15- to 30-minute period. Cardiovascular measurements were repeated 1, 2 and, sometimes, 3 hours after the end of the aniline infusion. In six experiments the aniline infusion was accompanied or preceded by the intravenous administration of the ganglionic blocking agent, pentolinium bitartrate (5 mg per kg). In four additional experiments the viscosity relative to that of dog plasma was measured at several erythrocyte concentrations before and after the infusion of aniline. An Ostwald viscometer was placed in a water bath maintained at a constant temperature (37° C) and the viscosity of the blood samples, also maintained at bath temperature, were measured using a driving pressure of 40 mm mercury.

Results

The cardiovascular responses in eight dogs to methemoglobinemia are illustrated in Fig. 1 and are summarized in Tables I and II. In every experiment the cardiac output increased as the arterial oxygen content was reduced by the formation of methemoglobin. The erythrocyte concentration, heart rate, arterial pressure, respiration rate, and oxygen consumption also increased, whereas the arteriovenous oxygen difference and total peripheral resistance decreased after the aniline. The changes in pulmonary arterial or right ventricular pressure were inconsistent. Several measurements of the arterial oxygen saturation after aniline showed values above 85%, which means that the residual hemoglobin was adequately saturated. Table II shows that along with the increased concentration of erythrocytes after aniline the apparent viscosity of the blood increased. This increase was, however, not different from that of normal dog blood with the same erythrocyte concentration. From Tables I and II it will be noted that although the apparent viscosity of the blood increased after aniline administration, the total peripheral resistance was decreased (which is another reflection of the elevated cardiac output).

In a series of six dogs which had received pentolinium bitartrate before administration of the aniline the relative increases in cardiac output from 81.5 ± 11.57 ml per minute per kg before, to 129.5 ± 17.72 at 1 hour and 142.3 ± 18.54 at 2 hours after, the aniline were not significantly different from those obtained at similar times in the series of eight unblocked dogs (see Table I). In this series the mean heart rate and erythrocyte concentration did not change after the administration of aniline, whereas the respiratory rate increased considerably as the arterial oxygen content was reduced.

TABLE I

Summary of changes* induced by methemoglobinemia

Period	Art. O_2 content (vol. %)	Cardiac output (ml/kg/min)	Erythrocyte conc. (%)	Mean art. press. (mm Hg)	TPR (PR units)	Heart rate (beats/min)	Oxygen consump. (cc/min)	Resp. rate (/min)
				A. In unblocked dogs				
Control	20.63 ±0.454	97.5 ±9.30	52.0 ±1.02	141.1 ±6.08	1.52 ±.159	160.4 ±11.13	89.4 ±9.16	15.0 ±4.44
1 hr after aniline	10.59 ±1.021	172.4 ±27.20	56.6 ±1.75	156.3 ±5.04	1.06 ±.177	192.9 ±17.29	104.5 ±7.43	28.4 ±3.87
2 hr	8.36 ±0.624	181.6 ±19.13	58.1 ±1.25	153.9 ±6.08	0.99 ±.108	207.7 ±11.57	105.3 ±8.36	27.0 ±3.63
3 hr	8.05†	186.0	61.7					
				B. In pentolinium-blocked dogs				
Preaniline‡	16.94 ±2.61	81.5 ±11.57	43.8 ±1.86	109.5 ±9.09	1.12 ±.314	122.0 ±10.95	63.5 ±14.49	20.5 ±4.64
1 hr after aniline	7.74 ±0.556	129.5 ±17.72	46.8 ±2.17	111.0 ±8.79	0.77 ±.219	123.5 ±6.18	70.8 ±15.07	43.8 ±6.87
2 hr after aniline	6.90 ±0.488	142.3 ±18.54	46.7 ±2.08	106.0 ±6.89	0.65 ±.114	123.0 ±10.25	71.8 ±15.10	43.8 ±6.16

*Mean ± S.E.M.
†Three experiments.
‡Mean of one control and two postpentolinium values.

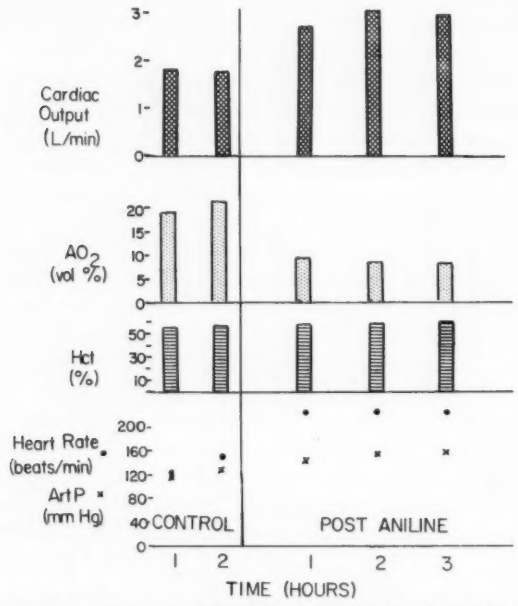

FIG. 1. Increase in cardiac output in methemoglobinemia. Dog 19 kg, male, anesthetized with sodium pentobarbital. The cardiac output (liters/minute), arterial oxygen content (volumes/100 ml blood), hematocrit level (% packed cells), heart rate, and mean arterial pressure (mm mercury) are shown. At the vertical line 150 mg per kg aniline dissolved in 0.9% saline were infused over a 14-minute period. Note the marked increase in cardiac output as the arterial oxygen content was reduced in spite of the increased blood viscosity as the hematocrit level rose from 54.5 to 58.0%.

TABLE II

Apparent viscosity* before and after aniline infusion

	No. of readings	Mean hematocrit level	Mean apparent viscosity
Control period	7	46.9%	2.48
After aniline	4	49.0%	2.55
Control period†	3	58.1	2.87
After aniline	4	57.1	2.83

*Viscosity relative to that of dog plasma (assigned value = 1).
†Packed cells were added to normal blood to make it polycythemic for comparison with postaniline values.

Discussion

The results show that aniline-induced methemoglobinemia lowered the arterial oxygen content within 1 hour and that the reduction was slightly greater at 2 hours. The suggestion (5) that the methemoglobin is formed not by aniline itself but by a metabolite, probably *p*-aminophenol, would account for its continuing appearance even though the aniline had been injected intravenously. The foregoing results are in agreement with those obtained by Clark

et al. (6), who administered aniline orally to three unanesthetized dogs and measured the cardiac output 3.5–9 hours later. They, too, noted that, although the oxygen capacity was markedly reduced, the arterial oxygen saturation of the active hemoglobin remained practically unchanged and there were no significant changes in total hemoglobin. In the present experiments the hematocrit level tended to rise after aniline. The reason for this hemoconcentration is not known, but because of it the effective viscosity increased. In spite of this increased viscosity, however, the cardiac output rose as the arterial oxygen content was lowered.

Richardson and Guyton (1) claimed that in anemia or polycythemia viscosity alone may have a major effect on cardiac output. That viscosity may be important in polycythemia is not questioned, but in anemia it is probably a secondary factor. In earlier experiments in which anemia was produced acutely by dilution of the blood with plasma substitutes it was found (7) that no marked increase in cardiac output occurred until the erythrocyte concentration was reduced below 35%. In acute normovolemic exchange anemia (blood withdrawn and an equal volume of dextran solution injected) consistent increases in cardiac output occurred only after the hematocrit level had fallen below 20% (8). Yet Levy and Share (3) found that the apparent viscosity of blood as measured in the hind limb was only slightly greater than that of plasma until the erythrocyte concentration exceeded 30%. Although in anemia the greatest changes in cardiac output do not occur until the erythrocyte level is reached at which the apparent viscosity is close to that of plasma, the present experiments show that the cardiac output can rise in response to a lowered arterial oxygen content in spite of increased viscosity of the blood.

That the increased cardiac output following aniline is related to the decreased arterial oxygen content rather than to a cardiotonic effect of aniline per se is likely for two reasons. Bonnet and Lelu (9) found that aniline was toxic to the frog heart, markedly increasing the chronaxie. More important, however, is the fact that the elevation of cardiac output in the present experiments is of the same magnitude as that observed when the arterial oxygen content is reduced by the production of acute anemia. In Table III the cardiac outputs and

TABLE III

Comparison of changes in cardiac output with reduced AO_2 in methemoglobinemia and anemia

Series	No. of expts.	Cardiac output* (ml/min/kg)	Art. oxygen content* (vol. %)
Aniline–methemoglobinemia	8	182 ± 19.1	8.36 ± 0.62
Plasma–anemia†	9	168 ± 12.2	4.10 ± 0.15
Dextran–anemia†	5	208 ± 7.9	4.17 ± 0.35

*Mean and S.E.M.
†Data taken from ref. 8.

arterial oxygen contents of the present aniline series are compared with those obtained in an earlier experiment (8) in which acute anemia was produced in dogs by the exchange of dextran or homologous plasma for whole blood. It can

be seen that, although the arterial oxygen content was not reduced as much in the aniline series as in the anemias, the cardiac outputs were not significantly different in the three types of experiment.

The results also show that the increase in cardiac output is not prevented by a dose of the ganglionic blocking agent, pentolinium bitartrate, sufficient to prevent an increase in heart rate in the present experiments and which, in earlier experiments, was shown to inhibit several important cardiovascular reflexes (10). It therefore appears unlikely that the sympathetic nervous system is indispensable in this response.

These results indicate that although in acute anemia the elevated cardiac output is accompanied by a reduction in the effective viscosity of the blood and in peripheral resistance, in methemoglobinemia the cardiac output rises even when the viscosity increases. It is, therefore, very unlikely that the response of the heart to a reduced arterial oxygen content is dependent upon changes in blood viscosity.

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THE ROLE OF ESTERASE INHIBITION IN TETRAETHYLPYROPHOSPHATE POISONING IN THE HOUSEFLY, MUSCA DOMESTICA L.[1]

D. STEGWEE

Abstract

The effect of tetraethylpyrophosphate (TEPP) was studied on the in vivo activity of different esterases in the housefly. TEPP was found to cause inhibition of the acetylcholinesterase and of the ali-esterase which hydrolyzes ethylbutyrate. The latter esterase could be selectively inhibited in vivo by treating the flies with tri-*o*-cresylphosphate (TOCP). Typical symptoms of organophosphorus poisoning developed only after TEPP when acetylcholinesterase was inhibited. Inhibition of this enzyme coincided with a rise of the level of acetylcholine in the insects. Treatment with TOCP caused a lowering of the level of acetylcholine. The insects became less sensitive to subsequent treatment with TEPP and in this case showed a lesser degree of accumulation of acetylcholine. The importance of acetylcholinesterase and ali-esterase in TEPP poisoning is discussed. It is concluded that the major biochemical lesion effected was the inhibition of acetylcholinesterase. Inhibition of the ali-esterase was not directly related to the toxic action of TEPP, although possibly it led to interference with the accumulation of acetylcholine resulting from the acetylcholinesterase inhibition.

Introduction

At present there is abundant evidence for the hypothesis that the insecticidal action of organophosphorus compounds is closely associated with their ability to inhibit the enzyme acetylcholinesterase (AChE) (1). This inhibition is believed to cause accumulation of acetylcholine (ACh) as was observed by Smallman and Fisher (2) for heads and trunks of houseflies and by Colhoun (3, 4) for nerve cords of roaches after treatment with TEPP. The excess ACh would appear to be responsible for the disturbance of nervous transmission, which leads to hyperactivity, prostration, and paralysis (4). As yet there is no evidence that interference with the ACh–AChE system is the direct cause of death in insects.

Criticism of the above hypothesis is based mainly on two observations. First, there is still no precise evidence that ACh is the substance responsible for synaptic and neuromuscular transmission in insects (3, 5, 6). Second, organophosphorus compounds are known to interfere with the action of other esterases besides AChE. Among these are esterases which are known to occur in insect tissues (7, 8, 9). Of special importance in this respect is the work of Van Asperen (10), who found a low degree of inhibition of AChE in contrast to a high inhibition of ali-esterase (AliE) in houseflies exposed to *O,O*-dimethyl-*O*-2,2-dichlorovinylphosphate (DDVP).

The present study is an attempt to evaluate AChE and AliE inhibition in the housefly during the early stages of poisoning with TEPP. Sublethal doses of this compound were used in order to study possible correlations between

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symptomatology and the degree of esterase inhibition. Some of the results have been published in a preliminary report (11).

Materials and Methods

Insect Material

Three-day-old female houseflies were used for all experiments. The insects were obtained from laboratory cultures reared according to the method of Fisher and Jursic (12).

Inhibitors

TEPP was obtained from the Delta Chemical Works Inc., New York, N.Y. It was purified by Dr. E. Y. Spencer from the Pesticide Research Institute, London, Ontario, according to the method of Hall and Jacobson (13) to meet the specifications set by these authors. For application to insects TEPP was dissolved in acetone; 0.05 to 0.20 μg in 2 μl was applied topically to the dorsal abdomen. For determinations of the in vitro inhibition of esterases by TEPP, aqueous solutions of the inhibitor were used.

TOCP, Eastman Kodak, Rochester, N.Y., practical grade, was used without further purification. For topical application to the abdomen of flies TOCP was dissolved in acetone and amounts from 10 to 200 μg in 2 μl were used. For injections a solution in olive oil was used. Amounts from 10 to 20 μg in 1 μl were injected into the thorax through the cervical membrane. Acetone solutions were used for in vitro determinations of esterase inhibition by TOCP.

Eserine sulphate, Nutritional Biochem. Corp., Cleveland, Ohio, was used for in vitro esterase inhibition studies only. Aqueous solutions were used in all experiments.

Determination of Esterase Activities

Determinations of esterase activities were performed in duplicate with homogenates of heads, thoraces, abdomens, and isolated thoracic ganglia of houseflies. The manometric method was used at a temperature of 25° C and pH 7.4. The reaction mixture contained $NaHCO_3$ 0.025 *M*, NaCl 0.085 *M*, KCl 0.002 *M*, $CaCl_2$ 0.009 *M*, enzyme, and substrate in water to a total volume of 2.7 ml. The gas phase consisted of 5% CO_2 in N_2. Readings were taken at 10-minute intervals for at least 40 minutes. The activities were calculated by use of the method of Aldridge *et al.* (14) and they were expressed as the amount of CO_2 evolved over a period of 30 minutes (b_{30}). Whenever necessary, corrections were made for non-enzymatic hydrolysis of substrate and for endogenous acid formation by the enzyme preparations in the absence of added substrate. The substrates used were acetylcholine, ethyl-*n*-butyrate, phenylacetate, and glyceryltriacetate (triacetin). With the exception of ACh, these esters were purified by redistillation. Details about the use of the various substrates for esterase determinations are given in the text.

Determination of Acetylcholine

Determinations of total ACh were carried out in duplicate with pooled samples of 30 heads and 30 thoraces of houseflies. The method of Lewis and Smallman (15) as modified by Colhoun and Gilleberg (16) was used. The tissues

were placed for 1 minute in a beaker containing 5 ml of boiling HCl-acidified frog Ringer's solution, pH 3.9. After cooling, the whole content of the beaker was transferred to a small glass homogenizer and homogenized for 1 minute. The homogenate was centrifuged at 4000 r.p.m. for 3 minutes. The supernatant was decanted and the residue resuspended in acidified frog Ringer's solution and again centrifuged. The combined supernatants were brought to pH 6.0 by the addition, with constant stirring, of 0.3 *N* NaOH. The volume was made up to 10 ml with frog Ringer's solution and divided into two 5-ml aliquots. One of these was brought to pH 11 with 0.3 *N* NaOH to destroy ACh, and after 15 minutes neutralized with 0.3 *N* HCl. This aliquot was termed the "blank".

For the assay of ACh the eserinized frog rectus abdominis muscle was used. The muscle was kept in aerated $NaHCO_3$-buffered frog Ringer's solution, containing 5 mg of eserine per liter. The ACh content of an extract was determined by comparing the contraction of the muscle upon addition of a measured volume of the extract with the contraction caused by the addition of an equal volume of the "blank" to which a known amount of ACh had been added. ACh values were expressed in terms of acetylcholine bromide.

The frog Ringer's solution used in the extraction procedure contained 7 g of NaCl, 0.14 g of KCl, and 0.12 g of $CaCl_2$ per liter of distilled water. The buffered Ringer's solution used in the ACh assay contained in addition to these constituents sufficient $NaHCO_3$ to give a pH of 7.3.

Results

The Effect of Sublethal Doses of TEPP upon Acetylcholinesterase Activity in vivo

A study was made of the toxicity of 0.10 μg of TEPP topically applied to female houseflies. The results showed that this toxicity was variable. After 1 hour 65% of the flies had become prostrate; the remaining 35% were still capable of co-ordinated movements. At 3 hours after treatment 46% of the prostrate flies had recovered. The final observed mortality after 24 hours was 30% of the flies treated. These observations showed that a number of flies treated with 0.10 μg of TEPP exhibited sublethal symptoms of TEPP-poisoning, in a manner similar to that described by Smallman and Fisher (2). These authors also demonstrated that the AChE of flies treated with a sublethal dose of TEPP was completely inhibited at the time of prostration. The enzyme recovered approximately 60% of its activity in the next 3 hours, coincident with the recovery of the flies from prostration. The rate of recovery of inhibited, i.e., phosphorylated enzyme seemed surprisingly rapid when compared with dephosphorylation rates reported for AChE in the American cockroach (17) and the rat (18). This created some doubt as to the validity of the initial inhibition values found by Smallman and Fisher (2). It is known that in vitro inhibition by excess inhibitor present in the tissues which are homogenized can obscure the true degree of in vivo AChE inhibition (Colhoun (4)). Van Asperen (10) showed that in homogenates of flies treated with DDVP this effect could largely be overcome when the homogenates were prepared in the presence of added ACh, which apparently protects the enzyme against inhibition. The protection of human erythrocyte AChE by ACh against inhibition

by TEPP was demonstrated by Burgen (19).

The following experiment was carried out in order to check with TEPP-treated flies the possibility of in vitro AChE inhibition by excess inhibitor during and after homogenization (Table I). Homogenates were prepared from

TABLE I

Estimation of the in vivo inhibition of acetylcholinesterase in homogenates of heads of houseflies, prepared 30 minutes after topical treatment with 0.1 μg of TEPP per fly

	Acetylcholinesterase activity*			
	Homogenates in saline		Homogenates in saline/ACh	
Source of enzyme	b_{30}	% inhibition	b_{30}	% inhibition
A. Control flies (two heads)	59	—	59	—
B. Treated flies (two heads)	15	75	45	24
C. Control one head + treated one head	28	24†	51	2†

*Substrate: acetylcholine at 1.7×10^{-2} *M*. Each figure represents the average of two separate determinations.
†$\frac{\text{Activity A} + \text{activity B}}{2} = 100\%$.

heads of flies 30 minutes after treatment with 0.10 μg of TEPP per fly. The medium used contained 8.65 g of NaCl, 0.23 g of KCl, and 0.34 g of $CaCl_2$ per liter, without or with added ACh to a concentration of 2.1%. Homogenates from heads of untreated flies served as controls. The degree of AChE inhibition found in homogenates prepared in the presence of added ACh was only one-third of that observed in homogenates prepared in the absence of ACh. Obviously the latter high inhibition was caused by the presence of excess TEPP, as is illustrated by the experiment in which AChE was determined in a mixture of equal parts of treated and control homogenates. Thus, it seems clear that after treatment with TEPP, an excess of this inhibitor can be present in the material to be investigated. Also, it is evident that this does not necessarily mean interference with the estimation of the in vivo AChE activity, provided the substrate-protection technique is used. This follows from the finding that in the mixture of homogenates prepared in the presence of added ACh, no appreciable inhibition was found. Therefore, this technique was adopted to determine AChE inhibition in the flies treated with 0.10 μg of TEPP, as mentioned in the beginning of this section.

The TEPP-treated flies were divided into three categories:

(1) flies which were prostrate and remained so for the duration of the experiment (19 hours);
(2) flies which had become prostrate but recovered between 1 and 3 hours after the application of TEPP;
(3) flies which did not become prostrate at any time during the experiment.

At chosen intervals samples were taken from each group of insects, and AChE inhibition determined in the pooled thoraces. In insects co-ordinated locomotion is largely independent of the brain and is controlled mostly by the

thoracic nervous centers. Therefore, it was felt desirable to examine the AChE inhibition in the thoraces. To enable a comparison with previous work AChE determinations were also carried out with heads of treated flies. The results of this experiment are given in Fig. 1.

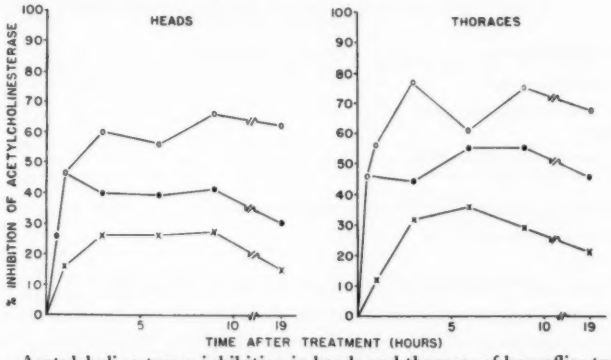

FIG. 1. Acetylcholinesterase inhibition in heads and thoraces of houseflies treated with 0.10 μg of TEPP.

○ Course of enzyme inhibition in flies remaining prostrate. ● Course of enzyme inhibition in flies recovered from prostration 2–3 hours after treatment. × Course of enzyme inhibition in flies which did not become prostrate.

Inhibition percentages are based on zero inhibition values found in untreated controls.

It should be noted that the inhibition of AChE at the time of prostration was low as compared with the inhibition found by Smallman and Fisher (2). Van Asperen (10) also found a low inhibition of AChE at the time of prostration in flies treated with DDVP, but showed that at the same time the AliE hydrolyzing methylbutyrate was inhibited to a large degree. This finding stimulated the investigation of the effect of TEPP upon the in vivo AliE activity.

The Effect of TEPP upon Ali-esterase Activity in vivo

Van Asperen (10) used methylbutyrate as substrate for determining the activity of AliE in the housefly. Accordingly, experiments were carried out with this substrate to determine the level of AliE inhibition in flies treated topically with 0.10 μg of TEPP. The procedure followed was essentially the same as that used for the determination of AChE activity in TEPP-treated flies. Methylbutyrate was incorporated into the homogenization medium so as to give a final concentration of 10^{-2} *M*. Flies were collected as soon as they had become prostrate. Their thoraces were pooled and homogenized and AliE and AChE activities were determined in the homogenates and in comparable homogenates of control flies. Similar determinations were performed 20 hours later with flies which had recovered from the treatment. The results are presented in Table II.

These observations, especially the high inhibition of AliE remaining after recovery of the flies, created some doubt as to the importance of AliE inhibition in TEPP poisoning. Therefore, it was decided to make a more detailed study of the esterases in the housefly, with special reference to the possible physiological effects of inhibition of AliE.

TABLE II

In vivo acetylcholinesterase and ali-esterase activity in houseflies after topical treatment with 0.1 μg of TEPP

Source of enzyme	Esterase activity*			
	Acetylcholinesterase†		Ali-esterase‡	
	b_{30}	% inhibition	b_{30}	% inhibition
Control flies, two thoraces	29	—	162	—
Treated flies, 1 hr after treatment, two thoraces	16	45	5	97
Treated flies, recovered, 20 hr after treatment, two thoraces	22	25	37	77

*Each figure represents the average of two separate determinations.
†Substrate: acetylcholine at 1.7×10^{-2} *M*.
‡Substrate: methylbutyrate at 10^{-2} *M*.

The Occurrence and Distribution of Esterases in the Housefly

The different sensitivity of esterases to carbamates on one hand and organophosphorus compounds on the other has been used by Mounter and Whittaker (20) as a means of classifying these enzymes. A distinction was made between cholinesterases (sensitive to both types of inhibitors), ali-esterases (sensitive to organophosphorus compounds only), and aromatic esterases (insensitive to either type of compound). On the basis of this principle, the occurrence of the different esterases in homogenates of heads, thoraces, and abdomens of houseflies was investigated. ACh, ethylbutyrate, phenylacetate, and triacetin were used as substrates. Eserine and TEPP were used as inhibitors.

The determination of the hydrolysis of ACh was carried out by the use of homogenates prepared with saline. These were placed in the main compartment of the Warburg flasks and ACh was added from the side-arm to start the reaction. The hydrolysis of triacetin was determined in the same way. The substrate was dissolved in water to give a final concentration in the reaction mixture of 10^{-2} *M*. For the measurement of ethylbutyrate hydrolysis the procedure was slightly different. Because of the low solubility of this ester in water it was dissolved in 50% ethanol. In order to minimize the ethanol concentration in the reaction mixture only 0.1 ml of substrate solution was used so as to give a final concentration of 1.7×10^{-2} *M* ethylbutyrate. To prevent undue evaporation of the substrate during gassing of the flasks, the substrate solution was placed in the stoppered side-arm of a double-arm Warburg flask. Phenylacetate has an even lower solubility in water than ethylbutyrate. Therefore, the method used by Metcalf *et al.* (9) for the determination of phenylacetate hydrolysis was adopted. The ester was dissolved in dioxane and 0.2 ml of this solution was placed in the main compartment of a standard Warburg flask together with 2 ml of the $NaHCO_3$–salts solution and 0.1 ml of 16% dioxane. The final phenylacetate concentration was 10^{-2} *M*. The homogenate was placed in the side-arm. The inhibitors were added to the flasks, which were then attached to the manometers, placed in the water bath, and gassed. The reaction was started 1 hour after the addition of the inhibitors.

Figure 2 shows the effect of various concentrations of eserine and of TEPP

upon the enzymatic hydrolysis of the above-mentioned substrates. From these results it was concluded that ACh was hydrolyzed only by AChE (cf. Augustinsson (21)), ethylbutyrate only by AliE, and phenylacetate and triacetin by AChE and AliE. The latter two substrates could also be hydrolyzed by aromatic esterases, which are known to occur in the housefly (9).

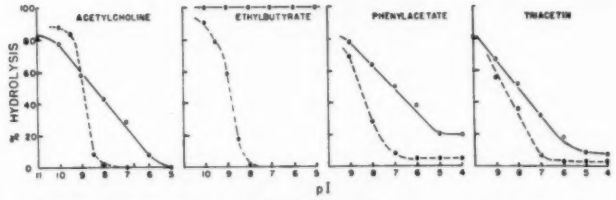

FIG. 2. The effect of eserine and TEPP upon the enzymatic hydrolysis of various esters by housefly homogenates. pI = − log concentration of inhibitor. The various substrates were used at 10^{-2} M.
○ Hydrolysis in the presence of eserine. ● Hydrolysis in the presence of TEPP.
Percentage hydrolysis was calculated on the basis of 100% hydrolysis in the absence of inhibitor.

The distribution of the different esterases in the housefly was studied with homogenates of heads, thoraces, and abdomens. Again, ACh, ethylbutyrate, phenylacetate, and triacetin were used as substrates. Eserine at 10^{-5} M and TEPP at 10^{-6} M were used for differential inhibition. The results are presented in Table III.

The distribution of AChE was as expected in view of the occurrence of ganglia of the central nervous system in the housefly. These are found only in the head and in the thorax. The distribution of AliE and of aromatic esterase was found to be the reverse of that of AChE. This suggested that the former esterases were mainly located outside the central nervous system. A determination of AliE and AChE in isolated thoracic ganglia showed that only 4% of the total thoracic AliE was found in the ganglion, as compared to 91% of the AChE.

Next, experiments were carried out in order to determine the number of AliE's involved in the hydrolysis of ethylbutyrate, phenylacetate, and triacetin. For this purpose the AliE activity with the individual substrates, as determined by using eserine and TEPP as differential inhibitors, was compared with the activity found with mixed substrates. The pertinent data are summarized in Table IV. The results showed that with none of the combinations of substrates used were the activities additive. Thus it appeared that only one AliE was involved in the hydrolysis of the three esters mentioned.

Using ethylbutyrate as substrate, the in vitro sensitivity of the AliE towards TEPP and TOCP was studied. Figure 2 already showed the sensitivity towards TEPP. The concentration of TEPP required to bring about 50% inhibition of AliE was 1.3×10^{-9} M. For the inhibition of AChE the same value was found and this result was in good agreement with that reported by Wolfe and Thorn (22). Mendel and Myers (23) demonstrated that TOCP acted as a selective inhibitor of mammalian serum AliE. It seemed worth while to investigate

TABLE III

Distribution of esterases in the housefly

Substrate	Esterase activity, b_{30}*								
	Acetylcholinesterase†			Ali-esterase‡			Aromatic esterase§		
	Head	Thorax	Abdomen	Head	Thorax	Abdomen	Head	Thorax	Abdomen
Acetylcholine 10^{-2} *M*	38±1.3	14±0.6	5±0.7	0	0	0	0	0	0
Ethylbutyrate 10^{-2} *M*	0	0	0	29±0.8	70±2.6	121±3.4	0	0	0
Phenylacetate 10^{-2} *M*	51±0.7	17±0.6	11±0.6	7±0.6	14±0.4	20±0.7	3±0.4	4±1.0	6±0.4
Triacetin 10^{-2} *M*	20±0.4	8±0.6	5±0.7	1±0.4	2±0.5	3±0.5	1±0.3	2±0.4	3±0.4

*Each figure represents the average of 5–10 determinations ± standard error.
†The part of the total esterase activity that was inhibited by eserine at 10^{-5} *M*.
‡The part of the total esterase activity that was inhibited by TEPP at 10^{-6} *M*, but not by eserine at 10^{-5} *M*.
§The part of the total esterase activity that was insensitive to TEPP and eserine at 10^{-6} and 10^{-5} *M* respectively.

TABLE IV

Hydrolysis of different substrates by housefly ali-esterase

Expt. No.	Source of enzyme	Substrates*	Ali-esterase activity† b_{30}
1	One whole fly	Methylbutyrate	220
		Ethylbutyrate	246
		Methyl- + ethyl-butyrate	240
2‡	One thorax	Ethylbutyrate	19
		Phenylacetate	12
		Ethylbutyrate + phenylacetate	19
3‡	One abdomen	Phenylacetate	16
		Triacetin	2
		Phenylacetate + triacetin	14

*Total substrate concentration: 10^{-2} *M*.
†Each figure represents the average of two duplicate determinations.
‡Esterase activity was determined in the presence of 8% dioxane.

whether this compound possibly also had a selective inhibitory effect upon housefly AliE. The hydrolysis of ethylbutyrate by a homogenate of thoraces of houseflies was measured after 1 hour incubation with TOCP at different concentrations. For comparison the effect of similar concentrations of TOCP upon the hydrolysis of ACh by a homogenate of heads of flies was determined. Table V shows the experimental results. The concentration of TOCP

TABLE V

The effect of TEPP and TOCP upon housefly esterases in vitro

Inhibitor	concentration,* *M*	Acetylcholinesterase† (three heads) b_{30}	Acetylcholinesterase† (three heads) % inhibition	Ali-esterase† (one thorax) b_{30}	Ali-esterase† (one thorax) % inhibition
TEPP	0	89	—	132	—
	10^{-10}	81	9	118	11
	3.3×10^{-10}	70	21	109	18
	10^{-9}	52	42	78	41
	3.3×10^{-9}	15	83	10	92
	10^{-8}	1	99	2	98
	3.3×10^{-8}	0	100	0	100
	10^{-7}	0	100	0	100
TOCP	0	102	—	66	—
	10^{-7}	—	—	65	2
	10^{-6}	—	—	63	5
	10^{-5}	104	0	55	20
	3.2×10^{-5}	—	—	30	55
	10^{-4}	105	0	9	86

*The enzyme preparations (homogenates) were incubated with the inhibitors for 1 hour at 25° C. Substrates used: acetylcholine and ethylbutyrate at 1.7×10^{-2} *M*. TOCP at 10^{-4} *M* was only partly dissolved in the aqueous medium.
†Each figure represents the average of two determinations.

required to give 50% AliE inhibition was calculated as 2.5×10^{-5} *M*. At a concentration of 10^{-4} *M* TOCP (saturated solution in equilibrium with suspension) the AliE was inhibited 86%. Under these conditions, however, AChE was not affected. Thus TOCP was shown to selectively inhibit the housefly AliE, which hydrolyzes ethylbutyrate.

The Effect of TOCP upon Ali-esterase and Acetylcholinesterase Activity in vivo

Experiments were carried out in order to establish whether TOCP was also

selectively inhibitory in vivo towards AliE in the housefly. It was thought that if this were so, it might elucidate the role of AliE inhibition in TEPP poisoning.

Upon determining the AliE activity in homogenates of whole flies which had been treated with TOCP, a difficulty arose in that it was not possible to use the device of substrate protection to avoid the occurrence of inhibition during and after homogenization. As was shown in Table III, the activity of AliE per fly was very high. Therefore, the effect of the presence of excess TOCP during homogenization was easily overcome by increasing the amount of medium, thereby diluting the homogenate. Thus the concentration of inhibitor was lowered to a level which caused no in vitro inhibition of the AliE in the homogenates. In all experiments this was checked by mixing a part of each homogenate with a homogenate obtained from normal flies and determining the AliE activity of the mixture.

Flies were treated with varying amounts of TOCP, either by injection or by topical application. After a given time the whole insects were homogenized in a volume of saline so as to give a homogenate containing the equivalent of one-half of a fly per milliliter. AliE and AChE activities were determined with 0.5- to 1.0-ml aliquots respectively, using ethylbutyrate and ACh as substrates. The results of these experiments are shown in Table VI. It appeared that TOCP

TABLE VI

The effect of TOCP upon housefly esterases in vivo

TOCP injected (μg)	Esterase inhibition after 2 hr*		TOCP topically (μg)	Esterase inhibition after 24 hr*	
	AChE† (%)	AliE‡ (%)		AChE† (%)	AliE‡ (%)
10	0	32	10	0	22
15	21	67	30	0	36
20	11	78	60	0	63
			80	0	70
			90	0	88§
			100	0	100
			200	0	100

*Each figure represents the average of two separate determinations on 30 flies.
†Substrate: acetylcholine 1.7×10^{-2} *M*.
‡Substrate: ethylbutyrate 10^{-2} *M*.
§Average of 10 determinations.

selectively inhibited the in vivo AliE activity, without affecting the activity of AChE. Of utmost importance was the fact that even when the AliE was completely inhibited, no symptoms of poisoning were observed. However, when these flies were treated with TEPP at 0.10 μg per fly, the insects developed typical symptoms of poisoning. Determination of the AChE activity at the time of prostration showed that this enzyme had become inhibited too. The pattern of esterase inhibition in these flies, treated with TOCP and 24 hours afterwards with TEPP, was not different from that found in flies prostrate after treatment with TEPP only and shown in Table II. There was, however, a distinct difference in the rate at which the symptoms developed. In the flies which had been pretreated with TOCP the rate of prostration by TEPP was

usually slower than in the controls. Also, the mortality after 24 hours tended to be lower in the pretreated flies.

Acetylcholine Levels in Houseflies Treated with TEPP and TOCP

Smallman and Fisher (2) found a correlation between the inhibition of AChE and the accumulation of ACh in houseflies treated with TEPP. Should such a correlation exist, then in flies treated with TOCP so as to inhibit AliE only, no accumulation of ACh should be found. Subsequent treatment of these flies with TEPP should result in an increase in the level of ACh.

Houseflies were treated topically with a dose of TOCP, previously determined to bring about complete inhibition of AliE without affecting AChE. Twenty-four hours later the insects were treated with a sublethal dose of TEPP. Control flies were treated with TEPP only. After 4 hours total ACh was determined in samples of 30 heads and thoraces as described under "methods". Since it was likely that any TOCP or TEPP present in the tissues was destroyed during preparation of the "blank", the effect of added TOCP and TEPP, in concentrations likely to be present in the extracts, was studied. No effect of these substances upon the contraction of the frog's rectus abdominis muscles was found.

The results, presented in Table VII, show the levels of total ACh found in the heads and thoraces before and after application of 0.20 μg of TEPP per fly. As predicted, no increase in ACh was found in the flies treated with 100 μg of

TABLE VII

The effect of TEPP and TOCP upon the level of total acetylcholine in the housefly

	Acetylcholine (μg per 30 flies)			
TEPP treatment	Controls	Number of determinations	TOCP-treated 24 hr after 100 μg/fly	Number of determinations
None	3.1±0.08	7	2.8±0.10	6
4 hr after 0.2 μg per fly	4.2±0.27*	4	3.1±0.21†	4

*91% prostrate.
†77% prostrate.

TOCP per fly only. Surprisingly, a significantly lower amount of ACh was found in these flies when compared to the level of ACh in untreated insects. Treatment of the TOCP-treated flies with TEPP resulted in an increase in ACh. However, this increase was significantly lower than that found in the flies which were treated with TEPP only.

Discussion

The results of the present study strongly suggest that the toxic effect of TEPP upon the housefly is primarily due to the inhibition of AChE. Like DDVP, TEPP brings about also a high degree of inhibition of AliE. However, the absence of symptoms of poisoning when the AliE is selectively inhibited by TOCP seems to exclude the possibility that AliE inhibition is of direct importance in TEPP poisoning in particular, and in organophosphorus poisoning in

general. Thus, the suggestions made by Lord and Potter (7), Hopf (8), Hopf and Taylor (24), and Van Asperen (10), viz. that inhibition of phenylesterase or AliE might be of greater importance than inhibition of AChE in poisoning with organophosphorus insecticides, seem to be untenable.

According to various authors AChE is present in large excess in nervous tissues and therefore toxic effects are not obtained below 80% inhibition of the enzyme (25, 26, 27). In the present work, however, it could be shown that the degree of AChE inhibition needed to cause prostration in the housefly was much lower. If the assumption is correct that the inhibition of AChE in the thoracic region of an insect is of major importance in disturbances of co-ordinated locomotion, the critical level of AChE inhibition in the housefly would appear to be approximately 50% (Fig. 1). However, it should be borne in mind that the inhibition values reported refer only to over-all inhibition. It seems possible that at critical sites in the nervous system, e.g., the synapses, the inhibition of AChE was much higher than the over-all percentage which was determined. There is no experimental evidence to support this hypothesis, but it offers an explanation for several seeming discrepancies. Thus it could explain the observation, also shown in Fig. 1, that previously prostrate flies recovered their mobility at a time when the inhibition of AChE was still increasing or at least remaining constant. Colhoun (28) showed that in the insect nervous system AChE is found in both synaptic and non-synaptic regions. There are strong reasons to assume that AChE exerts its function only at the synapses and neuromuscular junctions (see, e.g., Del Castillo and Katz (29)). When the over-all AChE inhibition is determined, this includes both "functional" and "non-functional" AChE, whose inhibition rates could well be very different. Repletion of inhibited synaptic AChE by non-inhibited enzyme from other parts of the nervous system could explain the recovery of the animals, whereas gradually increasing inhibition of non-synaptic AChE might account for the observed increase in over-all inhibition.

As was expected, accumulation of ACh occurred only when the AChE was inhibited, i.e., when the flies had been treated with TEPP. The finding, however, that the degree of ACh accumulation was significantly lower in the flies which had been pretreated with TOCP before the application of TEPP indicated that the relation between AChE inhibition and ACh accumulation is not a simple one.

The lower degree of ACh accumulation in houseflies treated with TOCP plus TEPP seemed to coincide with a slower rate of prostration and a lowered mortality, although it should be stated that the data obtained are at present insufficient to warrant any definite conclusion. The surprising fact remains that TOCP/TEPP treated flies do become prostrate at ACh levels which are not different from those found in the untreated controls (Table VII). One would tend to question the validity of determining the levels of total ACh, as has been done in the present work. It seems highly probable that it is in fact the level of free ACh which determines the occurrence of symptoms. Therefore, experiments are in progress now to establish the effect of TEPP and TOCP on the levels of free and bound ACh.

As to the question of how TOCP interferes with the accumulation of ACh, an explanation is not readily given. The data from Table VII would suggest that TOCP affected the synthesis of ACh. Whether the effect of TOCP upon ACh content and accumulation bears any relation whatsoever to its inhibitory effect upon AliE is also unknown. Of possible interest in this respect is the discovery made by Van Asperen and Oppenoorth (30) that in strains of houseflies which were resistant to parathion and diazinon the level of AliE activity was invariably lower than in susceptible strains. On the other hand, the observation that the AliE is located mainly outside the nervous system makes a direct relation less likely. A major difficulty is that the physiological function of the AliE is still obscure. Both the results obtained by Van Asperen and Oppenoorth (30) and those presented in this paper may constitute a step towards the ultimate elucidation of this problem.

For the present it seems justifiable to conclude that the inhibition of AChE is the major biochemical lesion in poisoning of the housefly with TEPP and that the symptoms are caused by the resulting accumulation of ACh. Inhibition of AliE is not directly involved but, at least in the case of the housefly poisoned with TEPP, seems to counteract to a certain extent the effect of the AChE inhibition.

Acknowledgments

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LACTIC ACID METABOLISM OF THE SUBMAXILLARY GLAND OF THE CAT[1]

B. C. R. Strömblad

Abstract

The decrease in oxygen uptake earlier found in the submaxillary gland of the cat after previous section of the chorda tympani is not associated with an increased lactic acid output. The differences in oxygen uptake found with different secretory rates from the normal gland are, at least partly, compensated for by an increase in lactic acid formation. At high concentrations of lactic acid in the blood the gland can metabolize lactic acid.

Previous work on the lactic acid metabolism of the submaxillary gland of the dog and cat has generally shown that an increased output of lactic acid from the gland accompanies secretion (4, 5, 8, 13, 14). It has therefore been concluded that lactic acid formation can furnish energy for secretion.

Some time after section of the chorda tympani the oxygen consumption of the gland both in the resting condition and during maximal stimulation by drugs is decreased (10, 11). This decreased oxidative metabolism could be compensated for by an increased anaerobic production of energy. The effect of section of the chorda tympani on lactic acid production in the gland has therefore been investigated.

Methods

Cats were used for the experiments. The right chorda tympani was cut, while the animals were under ether anaesthesia, 3 weeks before the experiment.

The animals were anaesthetized with chloralose after preliminary ether anaesthesia. The salivary ducts of the submaxillary glands were cannulated with glass cannulae which gave 40 drops/ml distilled water. The venous outflow from the gland was collected from a cannula in the external jugular vein; branches other than those from the gland were tied. The blood was collected in graduated cylinders and the time noted. Arterial blood samples, taken in the middle of the period of venous sampling, were withdrawn via a cannula in the femoral artery. Heparin was administered to prevent clotting. When the glands were stimulated, the sampling was begun not earlier than 2 minutes after the stimulus was applied, i.e. when a steady state could be expected to be attained. The secretory nerves were stimulated at different frequencies with supramaximal stimuli.

Estimation of lactic acid in saliva and in blood was carried out using a modification of the colorimetric method of Barker and Summerson (3) as described by Ström (9). All estimations were made in duplicate.

Calculations

The lactic acid output (or uptake) of the gland was calculated from the amounts present in the saliva, the arterial blood, and the venous outflow from

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the gland. When animals were in the resting state the volume of blood entering the gland was assumed to be the same as the measured volume leaving the gland. The volume of the lymph flow was disregarded. The error introduced thereby was considered to be very small since the lymph flow from the resting gland is less than 1% and in the secreting gland about 1% of the blood flow (1). In the gland secreting at a steady rate the volume of arterial blood entering the gland was expressed as the sum of the volume of venous blood and the volume of saliva. The latter volume was estimated by counting the number of drops of saliva falling from the cannula in the secretory duct.

Results

Resting Glands

The lactic acid outputs from the resting intact submaxillary gland of the cat and from the gland with the chorda tympani cut previously were small. The mean value and standard error for 15 intact glands was 2 ± 6 μg/minute and for four glands with the chorda cut was 18 ± 14 μg/minute.

Stimulation of Secretory Nerves

Stimulation of the chorda tympani at 20 c/second was found to cause an increased output of lactic acid from the gland. The figures obtained in six cats are given in Table I. In the table the lactic acid concentration in saliva and in

TABLE I

Lactic acid output from the submaxillary gland on stimulation of the chorda tympani

	Lactic acid concentration in mg per 100 ml			Lactic acid output in μg/min	
Cat No.	Arterial blood	Venous blood	Saliva	Saliva	Total from the gland (including saliva)
437	3.3	7.6	9.3	103	318
623	7.5	17.5	8.0	56	1096
745	2.2	6.0	4.8	24	141
695	7.0	11.6	14.4	65	335
679	6.0	11.2	15.1	83	423
617	3.7	11.0	9.8	59	589
Mean value	5.0	10.8	10.2	65	484

arterial and venous blood is also given. The concentrations in venous blood and saliva were very similar; in three of the cats the value for venous blood was the higher, while in the other three the reverse was true. The arterial concentration was always below that in venous blood and saliva.

In some experiments lower frequencies were used in order to get a lower rate of secretion. At low secretory rates there was little or no lactic acid in the saliva (Fig. 1) and the total output of lactic acid was not higher than at rest. On increasing the rate both the concentration in the saliva (Fig. 1) and the total output increased (Fig. 2).

Stimulation of the sympathetic nerve, at 20 c/second, also caused an increase in lactic acid production, although the increase was of a lower degree than that obtained on stimulation of the chorda. In four cats a mean value of 51 ± 7 μg/

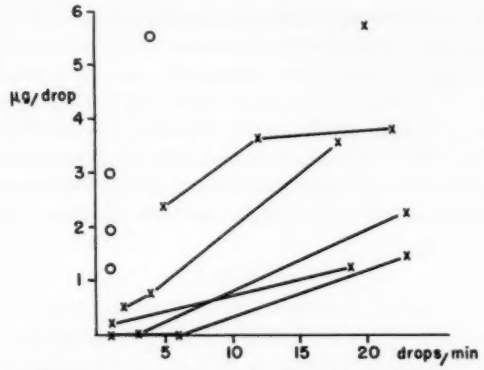

FIG. 1. Lactic acid concentration in saliva expressed in µg/drop (ordinate) at different secretory rates (drops/minute) plotted on the abscissa. Crosses: stimulation of the chorda (six cases). The values for a single animal are connected. Open rings: stimulation of the sympathetic (four cases).

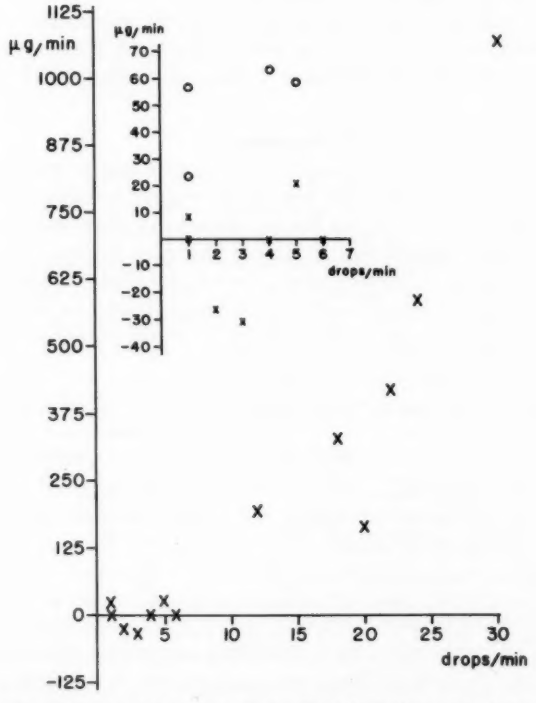

FIG. 2. Lactic acid output in µg/minute (ordinate) plotted against the secretory rate expressed as drops/minute (abscissa) on stimulation of the chorda tympani. The inserted figure is a magnification of a part of the main figure and shows also the values obtained with stimulation of the sympathetic in four cases (open rings).

minute was found. In these cases also the sampling was begun 2 minutes after stimulation had started. The secretory rate was therefore low; it is well known that in most cats the secretion on stimulation of the sympathetic rapidly declines with time. However, the lactic acid production in relation to the secretory rate was high when compared with the figures for lactic acid production from stimulation of the chorda that gave the same secretory rate (Fig. 2). A marked difference between stimulation of the sympathetic and of the chorda was also found when the lactic acid concentration in the saliva was compared at low secretory rates (Fig. 1).

Atropine

A dose of atropine which abolishes submaxillary secretion completely does not prevent an increase in oxygen consumption on stimulation of the chorda (2, 11, 12). It was thought of interest to determine if atropine could block the effect of stimulation of the chorda tympani on lactic acid metabolism. In six experiments atropine was given in small doses until the secretory effect of stimulation of the chorda was lost (0.25–0.40 mg of atropine i.v. was needed). No change caused by atropine in the lactic acid metabolism in the resting gland was found (5 ± 4 μg/minute of lactic acid produced).

On stimulation of the chorda an increased output was found in three of the cats while in the other three cats an uptake of lactic acid was found. Thus the stimulation after atropine did not seem to have any effect on lactic acid metabolism.

Increased Arterial Lactic Acid Concentration

In three cats the arterial lactic acid concentration was increased by infusing sodium lactate intravenously. The resting lactic acid metabolism in the gland was not significantly changed. In two of these cats there was an output of 19 and 13 μg/minute, respectively, while for the third cat there was an uptake of 6 μg/minute. A great difference from cats not given lactic acid was, however, noted when the chorda was stimulated. Instead of an output of lactic acid there was now a considerable uptake as can be seen in Table II, which also

TABLE II

Lactic acid metabolism in the submaxillary gland on stimulation of the chorda tympani. Raised arterial lactic acid concentration

Cat No.	Lactic acid concentration in mg per 100 ml			Lactic acid output in saliva in μg/min	Lactic acid *uptake* in the gland in μg/min (saliva not included)
	Arterial blood	Venous blood	Saliva		
768	43.5	36.0	17.3	147	900
695	75.0	66.5	44.4	200	650
734	75.2	62.6	59.8	269	391

shows the lactic acid concentrations of arterial and venous blood and of saliva. The concentration in arterial blood was higher than in the venous blood, in which, in turn, the concentration was higher than that in the saliva.

Effect of Previous Section of the Chorda Tympani

In four cats with the chorda cut 3 weeks earlier the output of lactic acid in

the resting state and during maximal rate of salivary secretion was estimated. The values found in the resting state have already been given. An intense stimulation in these cases was attained by injections of pilocarpine (20 μg) via the secretory ducts according to the technique described earlier by Emmelin, Muren, and Strömblad (7). By giving the drug via the duct the systemic effects of pilocarpine were, to a great extent, avoided. In Table III it can be seen that the total output of lactic acid from the glands, and also the output in saliva, was greater from the normally innervated gland than from its contralateral gland with the chorda cut.

TABLE III

Lactic acid output in μg/minute from the denervated and normally innervated submaxillary gland on stimulation with pilocarpine

	Chorda tympani cut 3 weeks earlier		Normally innervated glands		Denervated in % of normally innervated	
Cat No.	Saliva	Total from the gland (saliva included)	Saliva	Total from the gland (saliva included)	Saliva	Total from the gland (saliva) included)
651	164	323	260	650	63	50
623	158	273	248	388	64	70
624	180	422	300	1078	60	39
581	203	448	377	772	54	58
Mean value	176	367	296	722	60	54

Discussion

The present experiments show that the decreased oxidative metabolism of the submaxillary gland induced by section of its nerve (10, 11) is not met by increased lactic acid production either in resting or in stimulated tissue. On the other hand, the differences in oxygen consumption found for the normally innervated gland at different types of nerve stimulation (11) were counteracted by changes in lactic acid production. At high secretory rates less oxygen was consumed per volume of saliva secreted than at low rates, while the reverse was true for lactic acid output. Stimulation of the sympathetic caused less oxygen uptake, but greater lactic acid production than a stimulation of the chorda which gave a comparable secretory rate.

Several observations indicate that the lactic acid mechanism is not used preferentially by the gland for energy supply during secretion, but seems to be used only when the demand for energy is high and when the oxidative metabolism is insufficient. The lactic acid output was not increased at low secretory rates. Stimulation of the chorda did not cause an increase in lactic acid production comparable to that which occurred after administration of pilocarpine. This difference has also been noted by previous workers (13, 14) and attributed to less vasodilatation after pilocarpine than after stimulation of the chorda tympani. Stimulation of the sympathetic caused an increase in lactic acid output also at low secretory rates, presumably because of insufficient oxygen supply due to vasoconstriction. The low secretory rate obtainable during prolonged stimulation of the sympathetic does, however, not seem to be due

to lack of energy since the lactic acid output was much less than that obtainable on stimulation of the chorda. This seems true even if it is assumed that stimulation of the sympathetic does not excite all the secretory cells.

The results obtained in the experiments with atropine seem to tally with the assumption that lactic acid formation is used for energy only when the oxidative energy supply is insufficient, since no change in lactic acid output accompanies the stimulation of the chorda after atropine. There is, under these conditions, only a small increase in the oxygen demand.

The experiments in which the lactic acid content of the blood was increased showed that lactic acid can be metabolized by the gland. This conclusion is consistent with the results of Deutsch and Raper (6) obtained in experiments in vitro; lactate could maintain the oxidative metabolism in both resting and stimulated submaxillary tissue.

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PROTEINS IN FISH MUSCLE

16. ADENOSINETRIPHOSPHATASE ACTIVITY OF COD MYOSIN AND ACTOMYOSIN[1]

J. R. DINGLE AND J. A. HINES

Abstract

The adenosinetriphosphatase (ATPase) activities of actomyosin extracts of prerigor and postrigor cod muscle, and of myosins prepared from them by ultracentrifugation in the presence of ATP, have been measured at ionic concentration 0.1 and pH 7.3. The actomyosin ATPases were strongly activated by Mg^{++}, while those of the myosins were suppressed by Mg^{++}, in agreement with corresponding rabbit skeletal muscle proteins. Ca^{++} ion also moderately activated the actomyosin ATPases, but had little effect on the myosin ATPases. When actomyosin was precipitated by dilution in the presence of ATP and Mg^{++}, the characteristic activation by Mg^{++} was lost. The myosin ATPases were very labile.

Procedures for the direct extraction of myosin from mammalian tissue, such as the classical method of Szent-Györgyi (1) and its subsequent modifications, have not so far been successfully applied to fish muscle. A method due to A. G. Szent-Györgyi (2), based on the irreversible dissociation of actomyosin by potassium iodide, yielded a protein from cod muscle that appeared to be a modified myosin (3). Connell (4) reported the preparation of cod myosin by ultracentrifugation of a mixture of actomyosin and adenosinetriphosphate (ATP), following a method first used by Weber (5) for rabbit myosin. The sedimentation and diffusion characteristics of Connell's preparation indicated that it was analogous to mammalian myosin. We have confirmed the success of this procedure, but further evidence of this analogy could be useful. We have investigated the ATPase activity of such preparations, and also that of so-called actomyosin extracts of both prerigor and postrigor cod muscle.

It has been known for some time that both actomyosin and myosin from mammalian sources, principally rabbit, possess adenosinetriphosphatase activity (6). The enzymic activities of the two proteins differ, however, in the presence of magnesium or calcium ions at low ionic concentration ($\Gamma/2$) (7, 8, 9). For example, at $\Gamma/2 = 0.10$ or less, and pH 7.0, 10^{-3} M $MgCl_2$ activates actomyosin ATPase, but inhibits myosin ATPase. This difference in response to activators was used by Weber (5) to help identify her preparation of myosin. The results of the present investigation confirm that the material prepared by the ultracentrifugal procedure is analogous to mammalian myosin. Because of the great lability of cod myosin, already mentioned by Connell (4), it has not so far been feasible to carry out a thorough kinetic study of the enzyme reaction, and the present results must be regarded as hardly more than semiquantitative.

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Materials

Fillets cut from cod (*Gadus callarias*), obtained from the local inshore fishery, were used in the preparation of the protein extracts. The fish, which were of small to medium size (15 to 25 in.), were transported alive in small tanks to the laboratory, and unless used immediately, were held in a tank at approximately 2° C. The fish were either stunned, or anaesthetized with tricaine methanesulphonate (Sandoz Pharmaceuticals, MS 222), and filleted at once. Portions of fillets not used for immediate extractions were stored in covered beakers at 1° C. Extractions could thus be made on aliquots of the same fillet both before and after it had passed through rigor.

Adenosine-5′-triphosphate was obtained as the crystalline disodium salt from Schwarz Bioresearch Inc. Tris(hydroxymethyl)aminomethane ("Tris", Nutritional Biochemicals Inc.) was twice recrystallized from methanol (10) but the purification had no significant effect on the enzyme reaction, or on the stability of the myosin. The other materials, which were all of reagent grade, were used without further purification.

Methods

Samples cut from the middle of the fillet, and weighing 22.5 g, were extracted in a Waring blendor, fitted with a baffle plate (11) and operated at 90–100 volts. Two extraction procedures were used.

Procedure A

The sarcoplasmic constituents were removed by two 1-minute blendings with 0.05 *M* histidine, pH 6.6, using 425 ml and 375 ml of solution successively. This was followed by a 2-minute extraction of the fibrillar proteins from the residue with 375 ml of KCl–Tris solution (0.55 *M* KCl, 0.05 *M* Tris, pH 7.3). After each blending, the mixture was centrifuged at $1300 \times g$.

Procedure B

A total soluble protein extract was made by blending the sample for 2 minutes with 425 ml of the above KCl–Tris solution. In order to eliminate the water-soluble proteins, the extract was diluted 10-fold with water at 20° C, centrifuged at once for 8 minutes at $1300 \times g$, and the precipitated fibrillar proteins taken up in 3.0 *M* KCl–Tris, pH 7.3, to a final ionic concentration of 0.6. This process was repeated once.

Except for the use of water at 20° C in the precipitation steps, all operations were carried out in a cold room at 2° C, using precooled apparatus. The extracting solutions were degassed by suction, and partly frozen (about 5%) in order to reduce the temperature rise during blending. The use of water at 20° C for dilution greatly increased the speed of precipitation of the protein, and decreased the degree of centrifugation required. In this way, irreversible interactions between the precipitated proteins appear to be held to a minimum since there is little difference in the sedimentation patterns before and after precipitation (12).

In the prerigor extractions by procedure B, the final residue remaining undissolved was rather voluminous. The residue from procedure A was

usually similar, but in two cases it was very small. The residues from both types of postrigor extraction were always very small. The final yields of "actomyosin" were about 40% of the total protein N of the muscle in prerigor extractions, and in postrigor extractions about 65% by procedure A, and 50% by procedure B. The latter figure may be low because of incomplete precipitation. Allowing about 3% of total protein N in cod muscle for stroma (11) and 22% for water-soluble proteins (13), the maximum yield of salt-soluble ("actomyosin") proteins should be about 75% of the total muscle protein N.

After centrifuging at 2000×*g* to remove debris, 200 ml of the final extract or solution was mixed with 20 ml of an ATP–$MgCl_2$ solution (0.055 *M* ATP, 0.055 *M* $MgCl_2$, 0.55 *M* KCl, 0.05 *M* Tris, pH 7.3). The mixture was centrifuged at once for 1 hour at 42,040 r.p.m. in a Spinco model E ultracentrifuge, using a preparative rotor cooled to approximately 0° C. The supernate, which was usually water-clear, and presumably contained myosin, was easily decanted from the residue.

The myosin preparation was dialyzed in Visking cellulose tubing against two changes of 5 volumes of the KCl–Tris solution, $\Gamma/2 = 0.60$, pH 7.3, at about 2° C for a total of 24 hours. Similar dialysis of the untreated actomyosin extracts did not alter the enzymic characteristics, although the phosphate blank was decreased. This step was therefore usually omitted for actomyosin in order to make the enzyme assays within a few hours of preparation.

Reagents used in the enzyme reaction were dissolved in water and the pH adjusted to 7.0–7.5. The reaction mixture contained 0.05 *M* Tris buffer, pH 7.3, and the ionic concentration was adjusted where required by addition of KCl or NaCl. Other "standard" concentrations were: ATP, 0.0015 *M*; $MgCl_2$, 0.0015 *M*; $CaCl_2$, 0.01 *M*; these were found in separate experiments to yield maximum ATPase activity in actomyosin preparations at $\Gamma/2 = 0.1$. The protein concentration in the reaction mixtures varied from 0.02 to 0.05 mg N/ml. A mixture of appropriate amounts of the above reagents was made up to 25 ml. An aliquot of protein solution (2.5 ml) was diluted to 25 ml with water of the desired reaction temperature, and the resulting protein suspension added at once to the substrate mixture to start the reaction. It was found that if an aliquot of protein stock was added directly to an appropriate volume of substrate mixture, so that the dilution of the protein occurred in the presence of the ATP, the characteristic activation of actomyosin ATPase by Mg^{++} at low ionic concentration was lost.

The enzyme assays were carried out in a constant temperature water bath, stirring the mixtures with a submerged magnetic stirrer enclosed in a plastic bag. Aliquots of 1 ml were withdrawn at appropriate intervals, mixed rapidly with 9 ml of 10% trichloracetic acid, the precipitated protein removed by centrifuging, and 5 ml of the supernate analyzed for inorganic phosphate by the method of Rockstein and Herron (14). In separate experiments, the rate of non-enzymic hydrolysis of ATP in the assay procedure was found to be 0.07% per minute by the trichloracetic acid, and 0.09% per minute by the acid molybdate mixture. This was considered to be negligible for the purpose of this work.

Protein concentrations in the stock solutions were estimated by the biuret procedure of Snow (15). Interference from the Tris buffer was reduced to negligible levels by including it in the blank.

Sedimentation Analysis

Sedimentation analyses were made in the Spinco model E ultracentrifuge at 59,780 r.p.m. The rotor was precooled, and its temperature was maintained at approximately 0° C. The sedimentation constants (s') are given in Svedberg units for the conditions of the run, and are not corrected to water at 20° C.

Patterns found for the final "actomyosin" preparations by procedures A and B from the same sample of cod muscle both before and after it had passed through rigor mortis are shown in Figs. 1*a* and 1*b*. The total areas of the procedure A patterns were somewhat smaller than those of the procedure B patterns, even though the protein concentrations were adjusted to be equal; this may be due in part at least to a larger amount of rapidly sedimenting gel in the A preparations. It is also interesting that in the patterns of the prerigor B preparation (Fig. 1*a*, bottom) the amount of component III appeared to be unusually large relative to component II. The patterns resemble those reported by Ellis and Winchester (12) except that the peak due to their component I was now less prominent. It was found, however, that dialysis of an extract made by their procedure against the KCl–Tris system used in the present work led to a considerable decrease in the size of peak I.

The sedimentation patterns of the myosins prepared from the extracts just described are shown in Figs. 2*a* and 2*b*. Again, the total areas in the preparations derived from the method B extracts were somewhat larger than those from the A extracts, even though the total protein concentrations were equal. Each preparation had a small amount of rapidly sedimenting material, another component sedimenting just ahead of the main one, and a smaller amount of component IV. Some myosin preparations, however, appeared to be monodisperse. Examples made from both A and B extracts of a postrigor cod from a commercial source are shown in Fig. 2*c*. Only a trace of a rapid component could be discerned at an early stage of this run. The reasons for these differences are not known.

The sedimentation constant of the main peak in the myosin preparations did not appear to depend on whether NaCl–phosphate, used by Ellis and Winchester (12), or KCl–Tris was used as the solvent, but it did depend moderately on the protein concentration. Available data on the latter property are plotted in Fig. 3. The constant agreed well with that of component III ($s' = 3.1$–3.7) of the untreated "actomyosin" extracts. After an approximate correction to a liquid of the viscosity and density of water at 20° C, the sedimentation constant of the myosin at zero concentration became 6.8, in fair agreement with the value 6.43 found by Connell (4) and also with that of component III in the extracts of Ellis and Winchester (12).

Tests for Water-soluble ATPase

Kielley and Meyerhof (16) reported the preparation from rat muscle of a

PLATE I

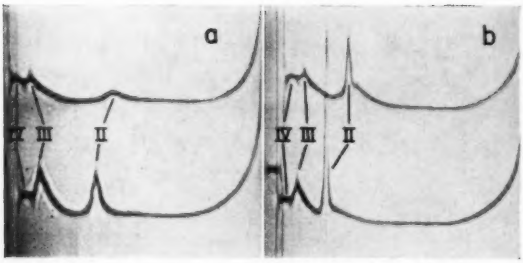

FIG. 1. Sedimentation patterns of actomyosin extracts of cod muscle. (*a*) Prerigor, 0.51 mg N/ml. (*b*) Postrigor, 0.69 mg N/ml. Upper trace, extract by procedure A. Lower trace, extract by procedure B. After 24 minutes at 59,780 r.p.m., $T \sim 0°$ C. Solvent, KCl–Tris, $\Gamma/2 = 0.60$, pH 7.3. Numbering follows Ellis and Winchester (12). Sedimentation to the right.

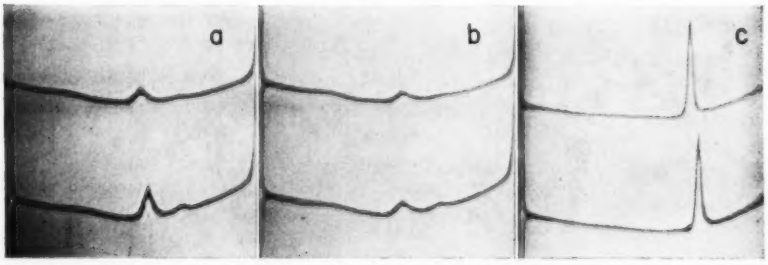

FIG. 2. Sedimentation patterns of myosin preparations from cod muscle. (*a*) Prerigor, 0.40 mg N/ml. (*b*) Postrigor, 0.47 mg N/ml. Upper trace, myosin from extract by procedure A. Lower trace, myosin from extract by procedure B. After 160 minutes at 59,780 r.p.m., $T \sim 0°$ C. Solvent, KCl–Tris, $\Gamma/2 = 0.60$, pH 7.3. (*c*) Postrigor. Upper trace, from extract by procedure A, 0.45 mg N/ml. Lower trace, from extract by procedure B, 0.38 mg N/ml. After 144 minutes at 59,780 r.p.m. Other conditions as in (*a*) and (*b*). Sedimentation to the right.

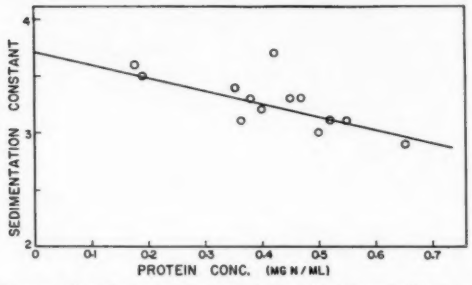

FIG. 3. Dependence of sedimentation constant of ultracentrifuge myosin on protein concentration. Constants are for the conditions of the run: $T \sim 0°$ C; 0.55 M KCl+0.05 M Tris, pH 7.3, or NaCl/phosphate, $\Sigma\, \Gamma/2 = 0.60$, pH 7.0.

Mg-activated ATPase, which was not myosin or actomyosin, but was associated with particulate matter remaining suspended after dilution of the preparation to low ionic concentration. To test for possible interference from a similar enzyme in our extracts, ATPase assays were made upon the following at $\Gamma/2 = 0.1$, in the presence of 0.0015 M $MgCl_2$:

(*a*) an extract of muscle made with histidine buffer, $\Gamma/2 = 0.05$, pH 6.6;

(*b*) the supernate remaining after precipitating protein, by a 10-fold dilution with water, from a KCl–Tris extract ($\Gamma/2 = 0.60$, pH 7.3) of the residue from extraction (*a*);

(*c*) the supernate remaining after two precipitations, by 10-fold dilution, of protein from a KCl–Tris ($\Gamma/2 = 0.60$, pH 7.3) extract of muscle. In each of these procedures, insoluble material was removed by centrifuging at $1300 \times g$. The samples were assayed without further dilution, but in no case was any activity detected. It may be that the white muscle of cod and other species is very poor in the Kielley–Meyerhof enzyme; Tonzetich and Kare (17) have reported that they could prepare a water-soluble ATPase from the red muscle of hen, but not from the white muscle.

ATPase Activities

The ATPase reaction curves for the actomyosin preparations whose sedimentation patterns have already been described are shown in Figs. 4*a* and 4*b*. The experimental points were corrected for phosphate blank (see below) before plotting. The shapes of the curves and the relative reaction rates calculated from them were similar for a number of other preparations studied, although the absolute rates varied from one extract to another.

After 5 minutes of reaction at 20° C, the amount of inorganic phosphate produced in the most active reaction cited in Fig. 4 corresponded to the hydrolysis of 65% of the ATP originally present. Even when this change in substrate concentration was allowed for, in this and other preparations, a linear relation for rate of hydrolysis could not always be demonstrated. It is not unlikely that some denaturation of the enzyme occurred during the reaction at 20° C or higher, but this was not fully investigated.

It was convenient to extrapolate the reaction curves to zero time to estimate the phosphate blank. This would not reveal the presence of any initial burst

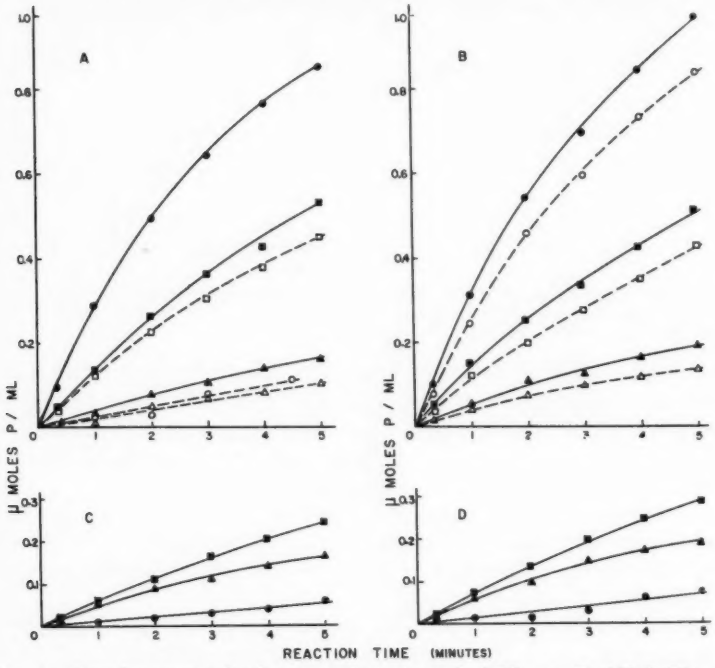

FIG. 4. Reaction curves of cod actomyosin and myosin ATPases. $T = 20°$ C; I/2∼0.1; pH 7.3. (A) Actomyosin, prerigor. (B) Actomyosin, postrigor. (C) Myosin, prerigor. (D) Myosin, postrigor. (In C and D, curves for myosin from procedure B extracts omitted for clarity.)

● ○ Activated by Mg^{++}, 0.0015 *M*. ■ □ Activated by Ca^{++}, 0.01 *M*. ▲ △ No activator. Full curves, extracts by procedure A. Broken curves, extracts by procedure B.

of reaction such as has been reported for actomyosin in 0.6 *M* KCl by Tonomura and Kitagawa (18). Direct checks of phosphate blanks in several runs agreed quite closely, however, with those found by extrapolation. The reaction rates were calculated from the amount of hydrolysis that occurred in the first 60 seconds of reaction as indicated by a smooth curve drawn through the experimental points.

The reaction curves found for the myosins (Figs. 4*c* and 4*d*) prepared from the above actomyosin extracts were similar in shape to those found for the actomyosins.

The rate constants calculated for all the reactions carried out in this representative experiment (No. 37) are given in Table I. The ATPase activities of the actomyosin extracts from postrigor muscle, prepared by both methods, and of that made by method A from prerigor muscle, were all activated by Ca^{++}, but were more strongly activated by Mg^{++}. On the other hand, that made by method B was activated by Ca^{++}, but not at all by Mg^{++}. All of the myosin ATPases were rather insensitive to Ca^{++}, and strongly inhibited by Mg^{++}. Moreover, the myosins were about twice as active as the actomy-

TABLE I

ATPase activities of actomyosin extracts and of ultracentrifuge myosin from prerigor and postrigor cod muscle ($\Gamma/2 \sim 0.10$; pH 7.3 (Tris); $[Mg^{++}] = 1.5 \times 10^{-3}$ *M*; $[Ca^{++}] = 10^{-2}$ *M*; $[ATP] = 1.5 \times 10^{-3}$ *M*. Activities in μmoles P/mg N/min)

Experiment No.	Extraction procedure	Activator					
		Mg^{++}	Ca^{++}	Nil	Mg^{++}	Ca^{++}	Nil
		Actomyosin			Myosin		
37 ($T = 20°$ C)	Prerigor, A	6.9	3.3	0.86	0.38	2.4	2.2
	Prerigor, B	1.0	4.9	0.90	0.78	1.8	2.4
	Postrigor, A	6.8	3.5	1.2	0.50	2.6	2.1
	Postrigor, B	7.2	3.5	1.2	0.44	3.0	2.1
35 ($T = 20°$ C)	Prerigor, A	8.0	3.6	0.91	—	—	—
	Prerigor, B	1.0	4.8	1.0	—	—	—
	Postrigor, A	8.4	4.0	0.95	—	—	—
	Postrigor, B	12.0	3.9	1.1	—	—	—
8 ($T \sim 13°$ C)	Prerigor, A	8.2	3.5	1.8	0	2.3	2.3
	Postrigor, A	7.3	3.3	1.3	0.1?	2.1	2.8

osins when no bivalent ion was added, a difference that seems too great to be accounted for by the presumed loss of actin from the myosin preparations.

Other data in Table I are included to illustrate reproducibility of activities. Not every preparation of myosin was active, but those that were active were comparable. The activities of actomyosin in the presence of Ca^{++} were reasonably constant, but those in the presence of Mg^{++} were rather variable. The latter were always greater than the Ca^{++} activities, however, except in the case of prerigor preparations by method B, which were always lower than the corresponding Ca^{++} activities.

In Table II are given some ATPase activities for actomyosin and myosin reported by others. Nearly all of the preparations were made from prerigor rabbit muscle, and the conditions of assay approximated those used in the present work.

It was possible to obtain identical reaction curves with the actomyosin preparations in duplicate assays made within an hour of one another. Runs made on successive days, however, showed a decreasing activity. The activity of the myosin preparations was even more labile; within 24 hours after dialysis (48 hours after preparation) the activity had usually fallen to zero.

In one experiment, samples of frozen cod that had been stored at −23° C for 13 days and for 7 months were extracted with 0.6 *M* KCl–Tris, pH 7.3. The yields of total extractable protein were 78% and 79% of the total protein N of the muscle, respectively, so that only a small amount of protein had become non-dispersible. A water extract of the 13-day sample gave a small activity (1.50 μmoles P/mg N/min) in the presence of Mg^{++}, which may have been due to a Kielley–Meyerhof enzyme. The "total" extracts of both samples exhibited strong activities of the actomyosin type, being strongly activated by Mg^{++} (Table III). It appears then that while the ATPase activities of the muscle proteins are labile once the proteins have been extracted, they are relatively very stable when left *in situ* in the frozen state. A similar stability

TABLE II

Representative ATPase activities of actomyosin and myosin from prerigor rabbit muscle, at $\Gamma/2 \sim 0.1$
(Activities given as μmoles P/mg N/min)

Reference	Activator						Conditions
	Mg^{++}	Ca^{++}	Nil	Mg^{++}	Ca^{++}	Nil	
	Actomyosin			Myosin			
25	1.08	3.3	0.60	—	—	—	0.04 *M* KCl } Tris, pH 7.5,
	0.39	3.0	0.77	—	—	—	0.08–0.10 *M* KCl } *T* = ?
8	1.44	2.1	1.14	0.60	1.80	0.18	0.07–0.10 *M* KCl; glycine, pH 7.0, *T* = 20° C
9	2.2	3.6	1.5	—	—	—	0.10 *M* KCl; 0.01 *M* histidine, pH 7.0, *T* = 21° C
5	1.3	2.6	—	0.07	2.7	—	0.06–0.08 *M* KCl; histidine, pH 6.6–6.9, *T* = 15–20° C
26	1.8	2.5	—	1.1	3.4	—	$\Gamma/2 = 0.08$; histidine, pH 6.7, *T* = 25° C
27	—	—	—	—	2.1 to 5.1	—	*T* = 25° C } $\Gamma/2 = 0.19$; 0.05 *M*
	—	—	—	—	0.39	—	*T* = 0° C } Tris, pH 7.2
28	—	—	—	—	3.3	—	0.004 *M* $CaCl_2$ } no buffer, pH 7.3,
					4.7		0.04 *M* $CaCl_2$ } 0.15 *M* KCl, *T* = 27.5° C
29	—	—	—	—	7.1	—	0.05 *M* KCl; 0.005 *M* $CaCl_2$; 0.02 *M* histidine, pH 7.6, *T* = ?

TABLE III

ATPase activities of actomyosin extracts from frozen cod muscle
(Activities given as μmoles P/mg N/min. Fish frozen postrigor)

Storage	Activator		
	Mg^{++}	Ca^{++}	
13 days at −23° C	10.8	3.2	Total extract, not precipitated
	10.5	3.0	Extract precipitated once
7 months at −23° C	—	3.2	Total extract, not precipitated
	8.0	1.8	Extract precipitated once

has been noted for the ATPase of frozen fish by Partmann (19) and Connell (20).

In another experiment, KI–myosin (3) was prepared from postrigor cod muscle, and a sample of ultracentrifuge myosin was prepared from the same fillet. The latter preparation exhibited normal ATPase activity, but the KI–myosin had no activity in the presence of 10^{-2} *M* Ca^{++}. Moreover, when the ultracentrifuge myosin was treated with 0.6 *M* KI, which was subsequently removed by dialysis, its ATPase activity was abolished. After addition of the KI, and before dialysis, the total ionic concentration was 1.2, but it is known from other work that similar treatment with KCl does not materially alter the ATPase activity of ultracentrifuge myosin. It appears, therefore, that if the protein prepared by treatment of actomyosin with KI is myosin, as claimed by Szent-Györgyi (2), then it must be a denatured form of that protein.

Discussion

A comparison of the representative data presented in Table I suggests that, as far as ATPase activity is concerned, ultracentrifugally prepared cod myosin is analogous with mammalian myosin prepared by the usual extractive methods. Not only was the characteristic inactivation by Mg^{++} at low total ionic concentration observed, but the magnitudes of the activities under various conditions agreed fairly well with those of the mammalian myosins. The results of the sedimentation analyses also tended to confirm the analogy. While in some of our preparations the heterogeneity suggested that the dissociation of the actomyosin had been incomplete, or that some aggregation of the myosin had occurred, the estimated sedimentation constant of the main component, $s_{20,w} = 6.8 \times 10^{-13}$, agreed with that of rabbit myosin (6.7–7.2) (21).

The case of the actomyosin extracts is rather less clear. Extracts of fish muscle made at moderate ionic concentrations, with or without prior removal of the sarcoplasmic constituents, consistently showed complex patterns in the ultracentrifuge. The very similar sedimentation behaviors of component III and of myosin strongly suggests that they are identical. If this is so, then the ATPase activities observed at low ionic concentration for "actomyosin" preparations may be the resultant of the activities of myosin and of true actomyosin(s) (presumably peaks I and II) (12). On the other hand, it is possible that lowering the ionic concentration to approximately

0.1 increases the proportion of actomyosin at the expense of myosin. Except for the work of Johnson and Landolt (22) and of Ellis and Winchester (12), the complex nature of actomyosin extracts has not usually been considered in studies of muscle proteins.

The low value for ATPase activity, in the presence of Mg^{++}, of prerigor actomyosin preparations made by procedure B was reproducible. Moreover, if any actomyosin extract, prerigor or postrigor, by method A or B, was added directly to the substrate mixture containing ATP and Mg^{++}, similarly low activities were consistently found. The common factor appeared to be precipitation of actomyosin in the presence of ATP and Mg^{++}. It is tempting to speculate that under these conditions the precipitated protein was largely myosin and that the characteristic suppression of myosin ATPase was being observed. This would follow from the dissociation of actomyosin that almost certainly occurs in the presence of ATP at $\Gamma/2 = 0.5$–0.6. On the other hand, this would not account for the other aberrations in the activities of actomyosin and myosin made from prerigor muscle by procedure B, apparent in Table I, and which appear to be reproducible from extract to extract. Nor was this hypothesis supported by an experiment in which an actomyosin extract of postrigor cod was mixed with ATP and $MgCl_2$ and then precipitated by dilution with water. Not all of the precipitated protein would redissolve in 0.6 *M* KCl but the ATPase activity of that portion which did dissolve was still enhanced by Mg^{++}.

In connection with these phenomena, the results of Saito and Hidaka (30) are interesting. Working with prerigor muscle, these authors claim to have prepared myosin A and myosin B by 20-minute and 24-hour extractions respectively, using a Greenstein-Edsall type solution. Both preparations were then precipitated several times to purify them. With both types of extract, the ATPase activity was enhanced about fourfold by Ca^{++} and reduced to about 50% by Mg^{++}. This behavior is similar to that of our prerigor "actomyosin" extract, using procedure B, rather than that of ultracentrifuge myosin. On the other hand, it is not clear from Saito and Hidaka's paper whether the reaction was carried out in 0.25 or 0.025 *M* KCl. The former appears to be the more likely, and if so, their results cannot be compared with ours because the influence of ionic strength on the reaction is known to be important in this range.

Saito and Hidaka (31) also report that while the activity of their myosin A and B fractions was rapidly reduced at elevated temperatures (37–45° C), only small deterioration was observed after storage for 48 hours at 1–3° C. This also agrees wth our experience with actomyosin preparations rather than with ultracentrifuge myosin.

It is usually stated that actomyosin is characteristically "strongly" activated by Mg^{++} at low ionic concentration (24). While this does seem to occur with cod actomyosin, it appears to be much less marked with mammalian actomyosin. It may be significant that the latter was usually prepared from prerigor muscle by a procedure analogous to our method B.

The original object of this work required that the ATPase activities be

studied at low ionic concentration, so that the system was necessarily heterogeneous. Because of this, the complex nature of the actomyosin extracts, and the extreme lability of the myosin, it did not seem worthwhile to carry out a thorough kinetic study at present. The effects of pH, temperature, and ionic strength will be presented in a later report, and a study of the ATPase activities of the fibrils is also being undertaken.

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CHANGES IN HEPATIC ENZYME LEVELS AFTER ADRENALECTOMY

II. GLUCOSE-6-PHOSPHATASE, GLUCOSE-6-PHOSPHATE DEHYDROGENASE, AND 6-PHOSPHOGLUCONATE DEHYDROGENASE[1]

J. S. WILLMER

Abstract

Adrenalectomy caused a decrease in the hepatic levels of glucose-6-phosphatase and glucose-6-phosphate dehydrogenase. Restoration to normal levels in rats which had been fasted for 18 hours was obtained by treatment with cortisone or corticosterone. In fed animals hydrocortisone and progesterone were also capable of maintaining an elevated level of glucose-6-phosphatase. Cortisone and corticosterone raised the level of this enzyme in fed sham-operated animals, but none of the other steroids showed this effect. The activity of 6-phosphogluconate dehydrogenase did not change significantly under the influence of adrenalectomy or individual steroid hormones.

A comparison is made between the effects of adrenalectomy and starvation upon the level of these enzymes in the liver during a 7-day period.

Introduction

The extent to which adrenalectomy and treatment with individual steroid hormones affected hepatic levels of phosphorylase, phosphoglucomutase, and phosphoglucoseisomerase was demonstrated in a previous communication (1). To complete the examination of enzymes concerned with glucose-6-phosphate utilization, glucose-6-phosphatase and two enzymes of the hexosemonophosphate shunt were studied under similar conditions.

The influence of adrenalectomy upon hepatic glucose-6-phosphatase has been examined (2) and shown to cause a decreased enzymic activity. By contrast, Ashmore *et al.* (3) were able to demonstrate an increase in the amount of glucose metabolized via the hexosemonophosphate shunt in the adrenalectomized rat.

In order that a more complete picture of the effects of adrenal corticoids might be obtained, these studies have been extended to include the influence of several biologically active steroid hormones upon hepatic enzyme levels in both adrenalectomized and sham-operated animals.

A comparison has also been made between the development of changes in enzymic activities in starved and adrenalectomized animals during a 7-day period.

Methods

The maintenance and hormone treatment of the animals was as previously described (1), all steroids being administered intraperitoneally at a dose level of 2 mg per day for a period of 6 days postoperatively.

Determination of Hepatic Enzyme Levels

Animals to be used for enzyme assays were starved for 18 hours prior to use, adrenalectomized animals being maintained on 1% sodium chloride. The

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activity of glucose-6-phosphatase was also determined in animals which had not been fasted.

Glucose-6-phosphatase

Glucose-6-phosphatase was assayed by a modification of the method of Cori and Cori (4). To tubes containing 0.1 ml 0.1 *M* sodium glucose-6-phosphate and 0.3 ml 0.1 *M* citrate buffer, pH 6.4, were added 0.1 ml aliquots of 5% liver homogenates. Incubation at 37.5° C was for 15 minutes when the reaction was stopped by the addition of 1 ml 10% trichloroacetic acid. After dilution to 10.0 ml and centrifugation, inorganic phosphorus was determined in 2.0 ml aliquots by the method of Fiske and Subbarow (5). A unit of activity is defined as the amount of enzyme which releases 1 μmole of inorganic phosphorus in 1 minute at pH 6.4 and 37.5° C.

Glucose-6-phosphate Dehydrogenase and 6-phosphogluconate Dehydrogenase

The method adopted for assaying these enzymes of the hexosemonophosphate shunt was essentially that of Glock and McLean (6), in which the rate of reduction of triphosphopyridinenucleotide is measured. The optimum pH for 6-phosphogluconate dehydrogenase of rat liver was found to be 8.5 and this modification was used in all assays. A unit of activity is defined as the quantity of enzyme which at 20° C and the specified pH, reduces 0.01 μmole TPN/minute, based on readings over the first 5 minutes.

Changes in liver cellularity upon starvation have been observed by Weber and Cantero (2), and might be anticipated also as a result of hormone treatments. Under the conditions of this investigation, however, the dry matter contents of livers from the 14 experimental groups were shown to differ by only 1.9%. The results of enzyme assays are therefore presented on a tissue fresh weight basis, as the mean of duplicate analyses from groups of eight animals.

Results

Adrenalectomy (Table I) caused a 22% reduction in the hepatic level of glucose-6-phosphatase in the fed animals. Restoration or maintenance of a normal enzyme level resulted from treatment with cortisone or corticosterone, while hydrocortisone and progesterone caused partial restoration only. Deoxycorticosterone and Reichstein's substance S were without effect in this respect. Cortisone and corticosterone also raised the glucose-6-phosphatase level significantly in sham-operated animals.

Starvation for a period of 18 hours did not change this picture (Table II), though the hepatic level of the enzyme was generally elevated, exceptions existing in groups of adrenalectomized rats treated with progesterone, deoxycorticosterone, and Reichstein's substance S. Adrenalectomy lowered the enzyme level in the liver by 26%, and restoration to normal occurred in groups treated with cortisone and corticosterone. Hydrocortisone, however, did not raise glucose-6-phosphatase above the level of the adrenalectomized control animals, nor did any of the hormones alter the enzyme activity of the sham-operated groups. As starvation did not appear to raise the level of

TABLE I

The influence of adrenalectomy and adrenal steroids upon hepatic glucose-6-phosphatase in fed animals[a]

Treatment	Sham-operated	Adrenalectomized
Control	882 ±12.0	690** ±18.0
Cortisone	1038** ±39.6	966[b] ±31.2
Hydrocortisone	912 ±27.0	810**[b] ±15.6
Progesterone	906 ±45.6	810**[b] ±13.2
Deoxycorticosterone	918 ±16.8	690** ±14.4
Reichstein's substance S	912 ±31.2	738** ±25.2
Corticosterone	960** ±21.6	948[b] ±30.0

[a]Enzyme activity expressed as units/g fresh liver/hour ± S.E.M.
[b]Significant at 1% level compared with adrenalectomized control group.
**Significant at 1% level compared with sham-operated control group.

TABLE II

The influence of adrenalectomy and adrenal steroids upon hepatic glucose-6-phosphatase in fasted animals[a]

Treatment	Sham-operated	Adrenalectomized
Control	1206 ±31.2	888** ±31.8
Cortisone	1122 ±27.0	1218[b] ±15.0
Hydrocortisone	1134 ±39.6	966** ±23.4
Progesterone	1152 ±37.8	792** ±25.2
Deoxycorticosterone	1116 ±55.2	732**[b] ±30.0
Reichstein's substance S	1098 ±33.6	792**[c] ±13.2
Corticosterone	1212 ±18.0	1122[b] ±16.8

[a]Enzyme activity expressed as units/g fresh liver/hour ± S.E.M.
[b]Significant at 1% level compared with adrenalectomized control group.
[c]Significant at 5% level compared with adrenalectomized control group.
**Significant at 1% level compared with sham-operated control group.

glucose-6-phosphatase in adrenalectomized rats treated with progesterone, deoxycorticosterone, or Reichstein's substance S, the results obtained with the latter two hormones indicated a significant decrease from the corresponding control values. In these respects the results are different from those obtained for fed animals.

Evidence for the effects of adrenal steroid hormones upon enzymes of the hexosemonophosphate shunt is presented in Table III. Glucose-6-phosphate dehydrogenase appears to be influenced in a similar manner to phosphor-

TABLE III

The influence of adrenalectomy and adrenal steroids upon hepatic glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase levels[a]

Treatment	Glucose-6-phosphate dehydrogenase		6-Phosphogluconate dehydrogenase	
	Sham-operated	Adrenalectomized	Sham-operated	Adrenalectomized
Control	153 ±3.8	101** ±3.4	348 ±25.2	304 ±15.4
Cortisone	186** ±7.2	128**[b] ±5.9	382 ±23.8	294 ±12.3
Hydrocortisone	173 ±16.4	118** ±8.4	392 ±23.0	320 ±11.5
Progesterone	130* ±7.8	100** ±6.0	339 ±16.7	313 ±12.9
Deoxycorticosterone	148 ±11.7	91** ±8.1	352 ±20.7	280 ±18.4
Reichstein's substance S	135 ±7.7	96** ±7.0	337 ±15.7	302 ±18.2
Corticosterone	164 ±10.5	134[b] ±8.1	391 ±21.7	301 ±15.0

[a]Enzyme activity expressed as units/g fresh liver ± S.E.M.
[b]Significant at 1% level compared with corresponding adrenalectomized control group.
*Significant at 5% level compared with corresponding sham-operated control group.
**Significant at 1% level compared with corresponding sham-operated control group.

ylase and phosphoglucoseisomerase (1) in that only glucocorticoids were capable of maintaining an enzyme level approaching the normal value. Although the values obtained for the adrenalectomized cortisone-treated rats were higher than the corresponding controls, they were still significantly lower than the sham-operated control animals. Among the sham-operated groups cortisone was the only hormone which evoked an increase in enzyme activity. In view of the significant effect of the glucocorticoids upon glucose-6-phosphate dehydrogenase it is surprising to observe the relative lack of effect of these hormones upon 6-phosphogluconate dehydrogenase. Adrenalectomy was not observed to exert any significant effect upon this enzyme, though in a comparison of corresponding adrenalectomized and sham-operated groups the mean levels obtained were higher in the latter animals. Changes in the hepatic level of this enzyme were also observed to be slight in lactating rats, in which glucose-6-phosphate dehydrogenase shows considerable alteration (7).

The results presented above were related to the sequential changes observed in adrenalectomized rats during a 7-day period, and in a group of animals starved to extinction. Glucose-6-phosphatase (Fig. 1) was observed to drop

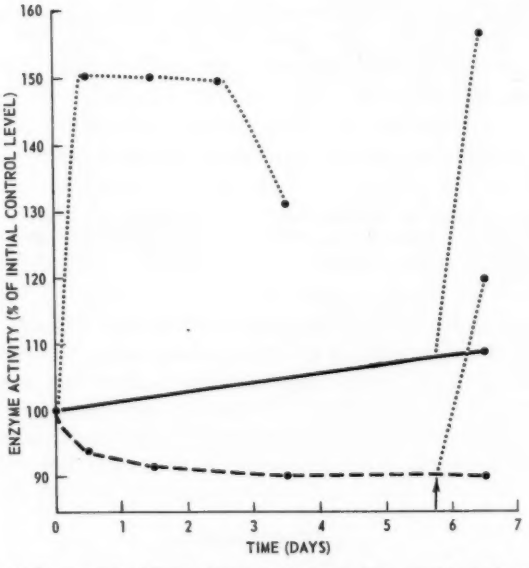

FIG. 1. The influence of adrenalectomy and starvation upon hepatic glucose-6-phosphatase levels. ●— — —● Adrenalectomized rats. ●– – –● Starved rats. ●———● Controls. ↑ Indicates commencement of starvation of control and adrenalectomized animals.

in adrenalectomized rats and reach a steady state 4 days after the operation. In contrast to this the enzyme level rapidly increased during the first 12 hours of starvation, plateaued for 2 days, and dropped sharply in the terminal stage of the experiment. Glucose-6-phosphate dehydrogenase (Fig. 2) decreased

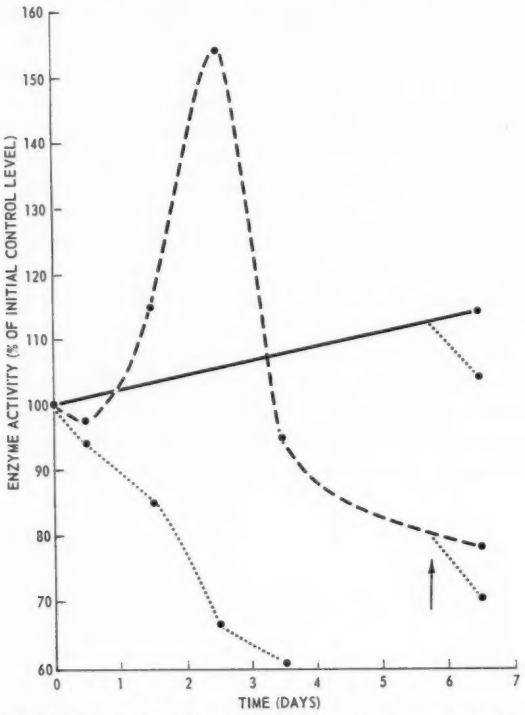

FIG. 2. The influence of adrenalectomy and starvation upon hepatic glucose-6-phosphate dehydrogenase levels. ●— — —● Adrenalectomized rats. ●- - -● Starved rats. ●——● Controls. ↑ Indicates commencement of starvation of control and adrenalectomized animals.

steadily during starvation but showed a pronounced increase in activity in adrenalectomized animals before finally dropping below the control level. The control values for both these enzymes increased during the experimental period, possibly due to the ingestion of glucose with the drinking water.

The levels of 6-phosphogluconate dehydrogenase under similar conditions showed only slight changes, though some loss of activity did appear on starvation.

Discussion

It was observed previously that adrenalectomy caused a decrease in the hepatic levels of phosphorylase, phosphoglucomutase, and phosphoglucose-isomerase (1). In general, it was also found that the enzyme level could be maintained close to the normal value by administration of one of the glucocorticoids, cortisone, hydrocortisone, or corticosterone, though in the case of phosphoglucomutase progesterone was also effective in restoring activity. Similar observations have been made with glucose-6-phosphatase and glucose-6-phosphate dehydrogenase. Adrenalectomy caused a 25–30% decrease in

activity, which could be restored by cortisone or corticosterone, and under some circumstances by hydrocortisone or progesterone. Certain of these findings are in close agreement with those of Weber and Cantero (2), who demonstrated a decreased level of glucose-6-phosphatase after adrenalectomy. Although cortisone was observed to raise the activity of this enzyme in normal fed animals, no effect was noted in fasted rats (8); nor were the influences of cortical hormones upon adrenalectomized animals studied. The effectiveness of the glucocorticoids in maintaining the hepatic enzyme levels is probably associated with their capacity to cause glycogen deposition, though it is difficult to integrate the effect of progesterone.

Ashmore *et al.* (3) in studying the metabolism of labelled glucose by rat liver slices observed an increase in the amount of glucose oxidized via the hexosemonophosphate shunt in adrenalectomized rats. As the animals used in these investigations were killed 4–6 days after adrenalectomy, the liver enzyme levels presumably corresponded approximately to the peak shown in Fig. 2. It is evident that under the conditions of our investigation such circumstances would not apply, as the peak activity is passed on the seventh postoperative day.

The influence of starvation upon glucose-6-phosphatase activity has been carefully studied by Weber *et al.* (2, 8–10). The observations presented in Fig. 1 substantiate their findings. The additional data concerning the changes associated with adrenalectomy indicate that starvation is apparently not responsible for the decreased enzyme levels, as, particularly in the case of glucose-6-phosphatase, starvation causes an intense stimulation of activity. These findings, in association with those of a previous publication (1), would indicate that removal of the adrenal glands causes an initial increase in the amount of glucose-6-phosphate metabolized via the Embden–Meyerhof pathway as revealed by an elevation of phosphoglucoseisomerase activity. This is succeeded by an increase in the activity of enzymes of the hexosemonophosphate shunt. The liberation of glucose by glucose-6-phosphatase drops steadily after adrenalectomy. However, 7 days after adrenalectomy, no one pathway of glucose metabolism appears to be more active than the others. The impairment of carbohydrate metabolism associated with adrenalectomy, as measured by changed enzymic levels, probably cannot be fully demonstrated by in vitro enzyme analyses which are obtained under optimum conditions, free from normal physiological regulating influences.

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EFFECT OF TESTOSTERONE AND GONADOTROPHIN INJECTIONS ON THE SEX ORGAN DEVELOPMENT OF ZINC-DEFICIENT MALE RATS[1]

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Abstract

Zinc-deficient rats, receiving a diet low in zinc (0.5 μg zinc/g), were injected subcutaneously with testosterone propionate or gonadotrophins for periods of 13 to 28 days.

The hormone injections in every case caused marked growth of accessory sex organs of zinc-deficient rats. The zinc concentrations of the large hormone-stimulated dorsolateral prostate glands were very low and similar to those of glands in untreated zinc-deficient rats. Testosterone propionate did not alter the testicular atrophy which had occurred before treatment. Gonadotrophin increased the rate of growth and possibly the rate of maturation of testes that were immature prior to treatment. When the testes had matured and were producing sperm before treatment, the tubular atrophy that is typical of zinc deficiency developed in spite of the injected gonadotrophins.

Testicular atrophy in zinc-deficient rats is probably due specifically to an inadequate supply of zinc to the testis. The availability of sufficient zinc for incorporation of high concentrations into sperm seems to be essential for the maintenance of spermatogenesis and the survival of the germinal epithelium. On the other hand, the reduction in rate of growth and development of immature testes and of all accessory sex organs in zinc-deficient rats is due mainly to an inhibition of pituitary gonadotrophin output.

Introduction

In a recent paper (1) we have reported the effect of dietary zinc deficiency on the growth, histological appearance, and zinc concentration of the reproductive organs in male rats. Increase in weight of organs and pituitary gland was greatly retarded in weanling rats fed a zinc-deficient diet for 6 to 8 weeks. Arrested spermatogenesis and atrophy of the germinal epithelium or delayed maturation were observed in the testes. Zinc concentration was reduced in testes, epididymides, and dorsolateral prostate glands. All changes except atrophy of testicular tubules were reversed by replacing zinc in the diet. If irreparable testis damage had occurred, the testes and epididymides did not regain normal zinc concentration after addition of zinc to the diet.

The reduced food intake and body growth of zinc-deficient rats suggested that restricted feeding of a zinc-supplemented diet might produce a similar effect on the genital organs. In rats given just sufficient food to keep their weight equal to that of zinc-deficient rats the limitation of the increase in weight of the accessory sex organs and pituitary glands was similar to that in zinc-deficient rats, but development and increase in weight of the testes were normal. The severe testicular damage found in zinc deficiency seemed to be due to the deficiency per se, but the reduced growth of the accessory sex organs was caused by the severe inanition.

Since, in simple inanition, retardation of sexual development is due to reduced

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output of pituitary and sex hormones, we have tested the response of the genital organs of zinc-deficient rats to administered testosterone propionate and gonadotrophins.

Methods

The method of preparation of the zinc-deficient diet, the care and maintenance of the animals, and the methods for histology and zinc analysis have been described previously (1).

Male hooded rats of approximately equal weight, aged 23–33 days, were fed the zinc-deficient diet for 6 to 8 weeks. They were weighed daily. When symptoms of zinc deficiency were apparent after 3 to 5 weeks (growth retardation, hair loss, etc.) unilateral orchidectomy was performed on each rat. The excised testis and epididymis were examined histologically and sometimes analyzed for zinc. In this way a measure was obtained of the condition of these organs before treatment began. The rats were then divided into the following groups receiving

(1) daily subcutaneous hormone injections:
   (*a*) 0.5 mg testosterone propionate in 0.1 ml olive oil, or
   (*b*) 20 International Units of Gonadogen (Upjohn Co. of Canada Ltd., from pregnant mare's serum) or 6 I.U. + 0.4 "activation units" FSH of Synapoidin (Parke, Davis Co. Ltd., combined pituitary and chorionic gonadotrophin); or
(2) daily oral supplements of 100 or 200 μg zinc as chloride; or
(3) no treatment.

After treatment for 2 to 4 weeks the rats were anaesthetized with ether and killed by exsanguination (heart puncture). The remaining testis and epididymis were removed and weighed. The testis, epididymis, dorsolateral prostate, and sometimes the ventral prostate and seminal vesicles were examined histologically. Dorsolateral prostates, testes, and epididymides were analyzed for zinc.

The response of non-genital tissue to hormone treatment was assessed by histological examination of the oesophagus and analysis of the ileum for zinc. Changes in the oesophagus due to zinc deficiency have been described by Follis, Day, and McCollum (2). We have found (3) that ileum zinc is reduced from the normal concentration of about 40 μg/g wet weight to about 20 μg/g in zinc-deficient rats.

The adequacy of the zinc-deficient diet was checked in each experiment by feeding two or three rats for the total feeding period on the diet supplemented with 100 or 200 μg zinc daily. These animals will be referred to as "diet controls".

Experimental

Testosterone Experiment

Seven weanling rats were fed on the zinc-deficient diet for 56 days. On the 35th day unilateral orchidectomy was performed for assessment of the condition of the genital tissues before treatment. Treatment was then begun. For the remaining 21 days four rats received testosterone propionate and three received

100 μg zinc daily. A "diet control" group of three rats received the zinc-deficient diet plus 100 μg zinc daily for 56 days to check adequacy of the diet.

Effect on General Condition of Animals

After 35 days the rats receiving the unsupplemented zinc-deficient diet had gained much less weight than the "diet controls" and they had symptoms of zinc deficiency such as alopecia and greying of the black hair (Table I).

TABLE I

Effect of testosterone propionate injections or zinc replacement on the body and organ weights and tissue zinc concentrations of zinc-deficient rats*

	Testosterone propionate 0.5 mg daily 21 days (4 rats)	Zinc replacement 100 μg Zn daily 21 days (3 rats)	Diet controls 100 μg Zn daily 56 days (3 rats)
Body and organ weights (means ± S.E.)			
Body, g			
Before treatment†	120 ± 4	112 ± 8	200 ± 6‡
After treatment	142 ± 10	222 ± 11	233 ± 8
Testis, mg			
Before treatment†	759 ± 63	738 ± 85	1374 ± 53‡
After treatment	564 ± 78	1008 ± 140	1540 ± 17
Epididymis, mg			
Before treatment†	155 ± 6	141 ± 16	323 ± 7‡
After treatment	251 ± 13	238 ± 8	443 ± 13
Dorsolateral prostate, mg, after treatment	231 ± 16	133 ± 28	170 ± 11
Ventral prostate, mg, after treatment	435 ± 24	207 ± 50	256 ± 28
Seminal vesicles, mg, after treatment	995 ± 36	302 ± 22	343 ± 7
Tissue zinc concentrations, μg Zn/g wet tissue (means ± S.E.)			
Testis			
Before treatment†	20.0 ± 2.3	20.0 ± 3.6	25.2 ± 0.2‡
After treatment	21.2 ± 3.7	17.9 ± 3.5	27.6 ± 0.5
Epididymis			
Before treatment†	16.9 ± 2.0	16.3 ± 0.9	49.1 ± 5.0‡
After treatment	17.1 ± 3.4	19.9 ± 3.4	39.6 ± 0.9
Dorsolateral prostate after treatment	46.7 ± 4.6	63.0 ± 5.5	151.7 ± 19.9
Ileum after treatment	19.8 ± 1.4	33.8 ± 2.4	34.9 ± 2.5

*Weanling rats fed zinc-deficient diet for 56 days. Testosterone or zinc treatment for final 21 days.
†Unilateral orchidectomy performed on the 35th day, before treatment was begun.
‡After 35 days on zinc-supplemented diet.

The rats that received testosterone for 21 days showed a slight initial gain in weight, but were beginning to lose weight when they were killed. External symptoms of zinc deficiency increased in severity during treatment. The zinc concentration of the ileum in testosterone-treated rats was below that in the "diet control" and "zinc replacement" groups and was similar to that in zinc-deficient rats. The histological appearance of the oesophagus was typical of zinc deficiency. Zinc replacement caused a dramatic gain in body weight, disappearance of external symptoms of zinc deficiency, and the return of the

TABLE II

Effect of gonadotrophin injections or zinc replacement on the body and organ weights and tissue zinc concentrations of zinc-deficient rats

	Experiment 1			Experiment 2			Experiment 3			
	Gonadogen 13 days 6 rats	Zinc replacement 13 days 6 rats	No treatment 5 rats	Gonadogen 28 days 4 rats	Zinc replacement 28 days 4 rats	No treatment 3 rats	Synapoidin 21 days 1 rat	Zinc replacement 21 days 2 rats		No treatment 1 rat
	Body and organ weights (means ± S.E.)							No. 1	No. 2	
Body, g										
Before treatment	94 ± 4	91 ± 5	92 ± 6	100 ± 6	101 ± 2	101 ± 1	154	129	146	132
After treatement	90 ± 4	141 ± 7	89 ± 6	96 ± 7	193 ± 14	102 ± 5	146	214	226	133
Epididymis, mg										
Before treatment	47 ± 11	34 ± 6	31 ± 4	73 ± 18	50 ± 10	31 ± 4	189	171	204	119
After treatment	115 ± 16	61 ± 7	31 ± 2	210 ± 26	178 ± 44	47 ± 11	235	304	356	130
Dorsolateral prostate, mg, after treatment	104 ± 11	17 ± 3	8 ± 1	236 ± 40	49 ± 16	9 ± 2	259	190	262	46
	Tissue zinc concentrations, μg Zn per g wet tissue (means ± S.E.)									
Testis										
Before treatment	17.7 ± 1.1	18.9 ± 0.9	18.7 ± 0.7	—	—	—	21.1	21.1	23.4	21.7
After treatment	15.8 ± 0.6	23.9 ± 0.6	19.8 ± 1.3	17.5 ± 1.0	24.4 ± 0.6	16.6 ± 1.2	13.2	24.7	26.7	11.0
Epididymis										
Before treatment	15.6*	14.1*	15.4*	—	—	—	26.2	29.0	24.1	23.4
After treatment	15.7 ± 2.6	20.8 ± 3.4†	—	16.8 ± 1.3	29.7 ± 1.2	24.5 ± 0.5	18.8	43.4	40.5	14.9
Dorsolateral prostate after treatment	27.0 ± 1.4	51.0*	32.0*	41.0 ± 1.5	57.3 ± 5.8	76.1 ± 15.0	—	83.5	92.2	40.6
Ileum after treatment	22.1 ± 0.8	35.7 ± 0.9	21.8 ± 0.7	20.0 ± 0.6	35.6 ± 2.0	21.9 ± 0.7	20.5	35.2	34.6	20.7

NOTE: Gonadogen is prepared from pregnant mares' serum by Upjohn of Canada Co. Ltd. Synapoidin is a combined pituitary and chorionic gonadotrophin prepared by Parke, Davis Co. Ltd.
*Pooled samples.
†Four rats.

zinc concentration of the ileum and the histological appearance of the oesophagus to normal.

Effect on Reproductive System

Before treatment was begun, after 35 days on the diet, testis and epididymis weights were much lower than those from the diet controls. The testes were mature, but some atrophy had taken place in about half the tubules examined. Some tubules appeared normal except that sperm production was negligible and histological examination of the epididymides showed few or no sperm. Diet control rats had normal mature testes with abundant sperm in the epididymides. Our normal rats become capable of fertilizing females at the age of 50–55 days.

After 21 days of treatment with testosterone or zinc the weights and histological appearance of the testes in both groups were not significantly changed ($0.1 > p > 0.05$). Epididymis weights increased in both groups but the organs did not attain normal size. In the testosterone-treated rats the weights of the accessory sex organs, including the dorsolateral prostate glands, were significantly greater than those in either the zinc-replacement or diet control groups. Mean weights of the accessory organs in the zinc-treated rats were not significantly different from those from the diet controls.

The concentration of zinc in testes and epididymides remained unchanged during treatment in both testosterone- and zinc-treated rats. Epididymis zinc concentrations were significantly lower than those of the diet controls in all cases. The zinc concentrations of the dorsolateral prostates were lower than those of the diet controls in both testosterone- and zinc-treated rats. The low concentration of zinc in the large dorsolateral prostates of rats treated with testosterone was similar to that observed in zinc-deficient rats (Table II).

Gonadotrophin Experiments

The preparation of the zinc-deficient diet and the care of the rats receiving the diet requires so much skilled assistance that we were unable to maintain more than 12 animals on the diet at one time. This led to difficulties in work with gonadotrophins because, although young animals were started on the diet at an apparently similar stage of development, by the end of the depletion period the sexual organs of all the rats were not equally mature. The condition of the organs at the start of treatment with gonadotrophin was determined by unilateral orchidectomy and the effects of the treatment varied according to the stage of development reached when treatment began. These circumstances resulted in only small numbers of animals being available in each group, which perhaps limits the value of the results.

Three experiments will be described in which testes had the following histological appearances before treatment began (1):

(*a*) immature, with no detectable cell damage,
(*b*) early mature, with little or no cell damage,
(*c*) mature, with little or no cell damage and numerous sperm in the epididymis,

(*d*) mature, with atrophy of germinal epithelium and arrested spermatogenesis.

In order to obtain rats with testes in condition (*c*) we used animals born of mothers that had been raised on the zinc-poor diet, plus 100 μg of zinc daily. After weaning, the young were kept on a similar regime for 49 days, when they ceased to receive supplemental zinc. It is very difficult to produce symptoms of zinc deficiency in normal mature animals because of the stored zinc in bone, hair, and other tissues, but these animals had been kept on a diet supplying just sufficient zinc to prevent development of deficiency symptoms. When the 100 μg Zn per day was withdrawn, symptoms appeared within 21 days, at which time unilateral orchidectomy was performed and treatment with gonadotrophin (Synapoidin) was begun.

Only five mature depleted rats reared in this way were available. One of these, treated with Synapoidin, accidentally received some zinc, as shown by zinc concentration of ileum and histological appearance of the oesophagus. This rat was discarded, leaving only one animal in the gonadotrophin-treated group. Nevertheless, the results of this experiment have been included because it will not easily be repeated.

Treatment with Gonadogen was continued in experiment 1 for 13 days and in experiment 2 for 28 days. In experiment 3 treatment with Synapoidin continued for 21 days (Tables II and III).

Effect on General Condition of Animals

In all gonadotrophin-treated rats, the body weights, the ileum zinc concentrations, and the histological appearance of the oesophagi were similar to those in the untreated zinc-deficient rats. Both treated and untreated rats appeared typically zinc-deficient. In the zinc-replacement groups, zinc concentrations of the ilea and the histological appearance of the oesophagi were similar to those found in rats receiving adequate zinc. Body weights were much increased after zinc replacement (1) but were still below normal in experiment 1, where the treatment period was only 13 days (Table II).

Effect of Treatment on Reproductive Organs

In every experiment, histological examination of the testes in the gonadotrophin-treated rats showed extensive interstitial cell proliferation. It is therefore assumed that in zinc-deficient rats gonadotrophin injections stimulated the production of androgens and hence produced rapid growth of the dorsolateral prostates and epididymes (Table II). The growth of accessory sex organs in the zinc-replacement groups of experiments 1 and 2, although significant, was much less than that observed in the Gonadogen-treated rats. In experiment 3 with older rats, accessory sex organ weights were similar in Synapoidin and zinc-treated rats. Testicular interstitial cell growth in all rats receiving zinc never seemed as great on histological examination as that observed in the gonadotrophin-treated animals.

In all experiments testis, epididymis and dorsolateral prostate zinc concentrations (Table II) were low in the gonadotrophin-treated rats and were nearly always similar to those found in the untreated zinc-deficient rats. In experi-

TABLE III

Effect of gonadotrophin injections or zinc replacement on the weight and histological appearance of testes in zinc-deficient rats

Histology before treatment	Expt. No.	Treatment time, days	Gonadotrophin treatment			Zinc replacement			No treatment		
			Weights, mg		Histology after treatment	Weights, mg		Histology after treatment	Weights, mg		Histology after treatment
			Before	After		Before	After		Before	After	
(*a*) Immature	1	13	292	793	Early mature	225	565	Immature	288	278	Immature
			177	562	Early mature	268	530	Early mature	270	391	Immature
			267	646	Early mature	139	480	Immature	224	273	Immature
			310	720	Early mature	236	500	Immature	274	319	Immature
									236	295	Immature
	2	28	362	818	Mature with atrophy	212	519	Early mature	266	521	Immature
									331	626	Mature with atrophy
									121	206	Immature
(*b*) Early mature	1	13	735	1001	Early mature	550	948	Early mature	No testes in this category		
			507	749	Early mature	317	642	Early mature			
	2	28	450	761	Mature with atrophy	389	1308	Mature	No testes in this category		
						463	1415	Mature			
						608	1190	Mature			
(*c*) Mature	2	28	1121	840	Mature with atrophy	No testes in this category			No testes in this category		
	3	21	1116	557	Mature with atrophy	1110	1325	Mature	938	581	Mature with atrophy
						1166	1230	Mature			
(*d*) Mature with atrophy	2	28	731	862	Mature with atrophy	No testes in this category			No testes in this category		

ment 2, epididymis zinc concentrations were significantly higher in the untreated rats than in the Gonadogen-treated group.

Zinc replacement invariably produced a small increase in the testis zinc concentrations and the means obtained after treatment were at a level normal for adult rats. Zinc concentrations of epididymides after zinc replacement were normal for adult rats only in experiment 3. Rather low values were observed for epididymis zinc in experiments 1 and 2, but this was not unexpected since the sperm contents of the epididymes were abnormally low. Sperm normally contains a very high concentration of zinc (4).

Dorsolateral prostate zinc concentrations, although always somewhat higher in rats treated with zinc than in those receiving gonadotrophins, did not reach normal levels.

Effect on Testicular Development

(a) Testes Immature before Treatment

The administration of gonadotrophins to zinc-deficient rats stimulated the growth and possibly the rate of development of testicular germinal epithelium in the absence of supplementary zinc when testes were immature. This was demonstrated (Table III (*a*)) in four rats having immature testes before treatment was begun (experiment 1). The testes of these animals grew and matured during the 13 days of gonadotrophin treatment in spite of the zinc deficiency. None of the untreated rats in experiment 1 had mature testes at the end of the experiment. Zinc replacement for 13 days produced only one mature testis from four rats having immature testes before treatment.

Once spermatogenesis had started subsequent gonadotrophin treatment did not prevent atrophy of the germinal epithelium in zinc-deficient rats. In experiment 2 (28 days treatment) the immature testis in one rat matured but eventually atrophied during the gonadotrophin treatment. Small numbers of sperm were found in the epididymis post mortem. In the untreated group of experiment 2, one rat had a testis which was mature with atrophy, while the other two rats had testes which were still immature at death. The one rat receiving zinc and having an immature testis before treatment had a recently matured testis after treatment.

In both experiments with Gonadogen and weanling rats, testis growth was considerable in immature organs during either gonadotrophin or zinc treatment but was always somewhat greater with the former. Testis growth was negligible in most untreated rats in experiment 1, and in experiment 2 it was less than in the treated groups.

(b) Testes "Early-mature" before Treatment

In two rats of experiment 1 (Table III (*b*)) having recently matured testes before treatment, these organs showed little or no increase in sperm production but did not atrophy during the 13 days of gonadotrophin treatment. Atrophy occurred in a similar testis in experiment 2, when the treatment was more prolonged (28 days).

Testes in the zinc-replacement groups showed a greater increase in weight than those in the gonadotrophin-treated groups. In experiment 1, there was

no change in the histological appearance of the testes or in the sperm content of the epididymides after 13 days of zinc treatment. In experiment 2, treatment with zinc for 28 days caused an increase in the number of sperm observed histologically in the epididymis over those seen in the organ removed before treatment. The testes were fully matured.

(c) Testes Mature before Treatment

Mature testes which, according to observations made on the specimens taken at the preliminary orchidectomy, had not begun to degenerate as a result of zinc depletion, were not maintained in a healthy condition by gonadotrophin treatment. In one rat receiving Gonadogen for 28 days in experiment 2 and in one rat receiving Synapoidin for 21 days in experiment 3 (Table III (*c*)), the testes were smaller and showed marked atrophy after treatment. Similar changes occurred in the one untreated rat from experiment 3. Zinc replacement to two rats in experiment 3 caused an increase in sperm production and possibly a slight increase in weight.

(d) Testes "Mature with Atrophy" before Treatment

Only one rat from the gonadotrophin treated group of experiment 2 was in this category. The testicular atrophy present before treatment was not arrested or alleviated during gonadotrophin treatment (Table III (*d*)). The testis removed after treatment was slightly heavier than the one removed before treatment.

To summarize, the administration of gonadotrophins to zinc-deficient rats produced a marked proliferation of testicular interstitial cells and growth of accessory sex organs. There was an increase in the rate of growth and possibly the rate of development of the immature testes, but in mature testes the injected gonadotrophins did not prevent the atrophy of spermatogenic tubules that occurred as a result of the zinc deficiency.

Discussion and Conclusions

In zinc-deficient rats, the dorsolateral prostate and other accessory sex organs grew rapidly under the stimulation of injected testosterone propionate or the endogenous androgens produced as a result of gonadotrophin injections. These results support the hypothesis that dietary zinc deficiency causes a reduction in pituitary gonadotrophin output and a consequent fall in androgen production. The previous food restriction experiments (1) indicated that this effect is due to the severe inanition that accompanies zinc deficiency rather than to the metal deficiency per se.

Zinc concentration was very low in the large dorsolateral prostate glands of hormone-treated rats. It appears therefore that zinc accumulation is not essential for the growth and development of this gland although normally zinc accumulates rapidly in the developing dorsolateral prostate glands of young rats (5). The function of the high concentrations of zinc found in the dorsolateral prostate glands of adult rats remains difficult to explain. Gunn and Gould (6) have recently shown conclusively that excision of the dorsolateral prostate gland, and hence the removal of high concentrations of zinc from the

seminal fluid, did not reduce fertility or fecundity in rats. They suggested that the high concentration of zinc in this gland may be "archaic", i.e. an evolutionary residue no longer of functional importance.

The results of the food restriction experiments previously reported (1) suggested that the testicular atrophy produced in zinc deficiency was due directly to an inadequate supply of zinc. The gonadotrophin experiments just described support this theory. The evidence cannot be considered conclusive for we have only a few observations and it is impossible to know to what extent the injected gonadotrophins, being foreign proteins and of arbitrary dosage, can be regarded as physiological replacement of the deficient endogenous pituitary hormones. Our results suggest that spermatogenesis cannot be maintained or testicular tubular atrophy prevented without the presence of adequate zinc, but that the decreased growth and rate of maturation of immature testes in zinc-deficient animals is probably due mainly to gonadotrophin deficiency and not specifically to zinc deficiency.

It appears that relatively large quantities of zinc are essential in the testis only after sperm production has started. This implies that the high concentrations of zinc in sperm (4) are attained during the final stages of sperm maturation. A similar suggestion was made by Wetterdal (7) as a result of his work on the rate of uptake of radioactive zinc by rat testis. The rapid attainment of high concentrations of zinc in testicular sperm would account for the small but consistent increase in the zinc concentration of rat testes occurring at sexual maturity (1).

The hormone experiments, together with the zinc deficiency and restricted feeding tests previously reported (1), have indicated that the only specific effect of dietary zinc deficiency on the male reproductive system in rats is to arrest spermatogenesis and cause atrophy of the germinal epithelium. Retarded growth and development of the genital organs appears to be due to a reduction in the output of pituitary gonadotrophins resulting from the severe inanition in the zinc-deficient rats. Thus we suggest that the presence of sufficient zinc for incorporation of high levels of zinc into spermatozoa is essential both for the complete development of individual sperm and for the maintenance of the germinal epithelium. On the other hand it has been shown that the accumulation of high concentrations of zinc in the dorsolateral prostate is not necessary for the survival or growth of this tissue.

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ISOMERIZATION OF VITAMIN A BY TISSUE HOMOGENATES[1]

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Abstract

Homogenates of liver, intestinal mucosa, and kidney were shown to catalyze the conversion of 11-*cis* to all-*trans* vitamin A. The liver contained the most active system and converted approximately 42% of the 11-*cis* in a 3-hour incubation. Intestinal mucosa and kidney preparations promoted conversions of 20% and 9% respectively. Only liver homogenates had an appreciable effect on the 13-*cis* isomer and caused a 15% conversion to all-*trans* vitamin A. The activity of all preparations was destroyed by boiling and renewed by the addition of unboiled homogenate. The extent of isomerization was dependent on the concentration of both homogenate and vitamin A. These results are compared to those found in vivo.

It has been shown (1, 2, 3) that when 11-*cis* and 13-*cis* vitamin A were given orally to rats there was some conversion to all-*trans* in the intestinal tract. A portion of these *cis* isomers was absorbed and stored in the liver but was rapidly replaced by all-*trans* vitamin A. It was not clear from these experiments whether the conversion to all-*trans* took place in the liver or elsewhere in the body. Hubbard (4) described an enzyme of cattle retinas and frog pigment layers which catalyzed the interconversion of all-*trans* and 11-*cis* retinene. This system was unable to convert isomers of vitamin A and was inactive with respect to isomers of retinene other than 11-*cis* and all-*trans*. No such system has been isolated from other parts of the body.

It is the purpose of this investigation to examine the activity of homogenates of liver and other tissues in the interconversion of 11-*cis*, 13-*cis*, and all-*trans* vitamin A.

Experimental

Two young male vitamin-A-deficient rats were used in each of the experiments described. They were killed by decapitation, and the livers, kidneys, and intestines removed. The livers and kidneys were blotted dry on filter paper until free of blood and then cut into small pieces. The first 24 inches of the intestines were cut into pieces about 4 inches long. These were slit longitudinally, laid flat on a Petri dish, and the intestinal mucosae were removed by gentle scraping with the back of a scalpel blade.

The tissues were homogenized by the method of Glover and Green (5) and fractionation was carried out at −5° C using a Lourdes refrigerated centrifuge model LR. The cell debris and nuclei were spun down at 750 *g* for 10 minutes, the supernatant poured off, and the pellet resuspended and spun again. This procedure was repeated. The supernatant and washings were then spun at 32,000 *g* for 60 minutes; this centrifuging caused a composite mitochondrial–microsomal pellet to form. In preliminary experiments with liver tissue,

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activity was found in all the cell fractions and, in the experiments reported here, the combined mitochondria–microsomes were used.

The pellet was suspended in 40 ml of the medium described by Krebs (6). A 5-ml aliquot of the mitochondrial–microsomal suspension was pipetted into a 50-ml Erlenmeyer flask and 9 ml of the medium and 1 ml of a water dispersion of vitamin A were added. Dispersions of all-*trans*, 11-*cis*, and 13-*cis* vitamin A were prepared to contain 1500 I.U./ml as previously described (3) except for the omission of tocopherol. The flasks were placed on a mechanical shaker and incubated in the dark at 37° C. In preliminary experiments incubation periods of 3, 24, and 48 hours were used. After 3 hours little change occurred and the results reported here are all based on a 3-hour incubation. After incubation, 15 ml of ethanol were added and the samples were stored in the dark at −20° C until analyzed.

Vitamin A was extracted with two 50-ml portions of petroleum ether and determined with the antimony trichloride method. The method of Robeson and Baxter (7) which distinguishes between "slow-acting" (11-*cis*, 13-*cis*) and "fast-acting" (all-*trans*, 9-*cis*) isomers was used to estimate 11-*cis* and 13-*cis* content. In this paper it has been assumed that all-*trans* was the only "fast-acting" isomer formed in appreciable amounts. The results of Hubbard (4) and Plack (1) indicate that this is probably the case. With each experiment at least two incubations were made in which 5 ml of medium was added to the incubation flask in place of the mitochondrial–microsomal suspension to give a value for spontaneous isomerization.

Results

Table I shows the results obtained with the various tissues. Each value is the mean of two separate incubations and determinations and was calculated by correcting the percentage *cis*-isomer content of each incubate by the appropriate blank value and subtracting the result from 100. Liver tissue converted both 11-*cis* and 13-*cis* vitamin A to the all-*trans* form, 11-*cis* to the extent of 37% to 54% and the 13-*cis* isomer from 13% to 17%. From 6% to 7% *cis* isomer was formed by incubation of all-*trans* vitamin A with liver homogenates. Intestinal mucosa converted 11-*cis* to the all-*trans* form to the extent of about 20% but had little effect on the 13-*cis* and all-*trans* vitamin A. Kidney tissue was active only in the conversion of 11-*cis* to all-*trans*, which it did to the extent of 8% to 10%.

The data of Table II indicate that boiled liver homogenates lost completely their ability to catalyze the conversion of 11-*cis* and 13-*cis* isomer of vitamin A to the all-*trans* form. When fresh tissue was added, the usual degree of conversion was obtained. The slight conversion of all-*trans* to a *cis* isomer was not influenced by heating the homogenates.

The effect of varying the concentration of both cell suspension and the vitamin A is illustrated in Table III. The conversion of 11-*cis* to all-*trans* vitamin was increased by increasing the cell concentration or by decreasing the vitamin A. Decreasing the cell concentration or increasing the vitamin A had the opposite effect.

TABLE I

Isomerization of vitamin A by rat tissue homogenates

Tissue	Expt.	Isomer incubated: 11-*cis*	13-*cis*	All-*trans*
		Conversion to all-*trans* in %		
Liver	1	38	—	—
	2	43	—	—
	3	36	—	—
	4	54	—	—
	5	40	15	−6
	6	49	17	−7
	7	37	15	−6
	8	—	13	—
	Mean	42	15	−6
Intestine	1	19	0	−2
	2	20	5	−2
	Mean	20	3	−2
Kidney	1	10	0	−4
	2	8	0	−1
	Mean	9	0	−3
Blank	Mean of all experiments	0	3	0

TABLE II

Effect of boiling on the activity of liver tissue in the isomerization of vitamin A

Isomer incubated	Experiment	Conversion to all-*trans* in %: Boiled homogenate	Boiled homogenate + fresh tissue
11-*cis*	1	0	43
	2	0	44
	3	0	48
	4	0	51
13-*cis*	3	2	40
	4	0	12
All-*trans*	3	−3	− 4
	4	−8	−10

TABLE III

Effect of liver tissue and vitamin A concentration on the isomerization of 11-*cis* to all-*trans* vitamin A

Substrate units of 11-*cis**	Experiment	Cell concentration: *C*†	*C*/5†
		Conversion to all-*trans* in %	
2000	1	48	27
	2	38	29
1000	1	61	37
	2	48	33
500	1	57	53
	2	59	32

*Determined by $SbCl_3$ reaction.
†C = concentration normally used.
C/5 = 1/5 normal concentration.

Discussion

These experiments demonstrated that certain tissues were capable of catalyzing the conversion of 11-*cis* and 13-*cis* vitamin A to the all-*trans* form. This activity was destroyed by heat and the extent of the conversion achieved depended on the concentration of both tissue and substrate. The maximum isomerization observed after 3 hours of incubation in the dark was the 42% conversion of 11-*cis* to all-*trans* vitamin A by liver tissue although this was surpassed in individual experiments especially when the cell-to-vitamin ratio was increased. Intestinal mucosa and kidney tissues were about one-half and one-fifth as active as liver tissue in this respect. The 13-*cis* isomer was more stable and was not converted to all-*trans* by any tissue except liver, which did so to the extent of only 15%. These results agree with those of Hubbard (4) in that the greatest conversion was that of 11-*cis* to all-*trans* vitamin A. However, Hubbard's enzyme, isolated from eye tissue, was completely inactive in the isomerization of vitamin A and required retinene as substrate. There is no information concerning the activity of liver tissue in the interconversion of isomers of retinene.

Previous experiments (2, 3) with the intact rat indicated that the more potent 13-*cis* isomer was more fully converted to all-*trans* vitamin A in the intestinal tract than was the 11-*cis* isomer. The rate at which these isomers disappeared from the liver was not notably different. Thus the in vivo and in vitro results do not agree on the extent to which these two *cis* isomers were converted to the all-*trans* form. The results with tissue homogenates are in line with the fact that the 11-*cis* isomer is the most unstable, thermodynamically, of the vitamin A isomers. Biological potency does not seem to be related to the ease with which *cis* isomers are converted to *trans* in vitro and other factors undoubtedly influence the results observed in the intact rat.

Acknowledgment

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STEADY-STATE OXIDATION OF GLUCOSE IN THE DIABETIC RAT[1]

DOROTHY S. DOW AND C. E. ALLEN

Abstract

The single intraperitoneal injection of a labelled glucose load has been shown to maintain the specific activities of blood glucose and expired CO_2 at constant levels for extended periods of time. These plateaus have been established in both the fed control and the fed diabetic rat. The results have been interpreted as indicating the existence of a steady state in the labelled system under study.

Measurements have been made of rates of oxidation of glucose-1-C^{14} and glucose-6-C^{14} in diabetic and control rats during this steady state between the specific activities of blood glucose and expired CO_2. The results indicate no reduction either for the glycolytic or for the phosphogluconate pathway in the diabetic. No change in the ratio between the oxidative rates for the two pathways has been found.

Introduction

Many workers have shown that when control or diabetic rats are given a single intravenous injection of labelled glucose, the specific activity of plasma glucose declines exponentially, because tagged sugar is progressively replaced by relatively non-labelled glucose (1–3). Concurrently with this, the curve for the specific activity of expired CO_2 at first rises gradually and then, after reaching a peak at approximately one hour, declines with a slope somewhat less than that for glucose. As pointed out by Stetten *et al.* (4), the contour of this curve for the specific activity of CO_2 is incompatible with the assumption of a single pool model which was made by earlier workers (1, 5). Constant intravenous infusion of C^{14}-glucose produced a continuous but varying increase in the specific activity of the expired CO_2. Consequently, using this technique, an arbitrary time for comparison of specific activities had to be selected (4). Recalculation of earlier data, according to the formula describing classic parent–daughter relationship in radioactive decay in a two-pool system, indicated that the single-injection and constant-infusion methods for studying glucose oxidation rates produced comparable results (6). But the above classic formula assumes that enrichment and dilution of the second pool are accomplished without entrance of diluting material except from the first pool. Since this is not so for respiratory CO_2, Baker *et al.* (3) and Shreeve *et al.* (7) employed a technique which involved comparison of the glucose curve maximum with the maximum value for a theoretical curve for glucose which assumed glucose to be the sole source of CO_2 (the 100% CO_2 curve). The value at the maximum was compared with the glucose curve value at the same time. However, as these workers pointed out, an appreciable delay in the intermediate pathways would tend to shift the curve for the specific activity of CO_2; thus the maximum and the contour of the curve would be altered from that of the classic two-pool system. Such a delay, indeed, is obvious in the relationship between the two specific activity curves for glucose

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and CO_2; the relationship is compatible with that of a two-pool system, each pool with a turnover time of appreciable magnitude.

From the foregoing it is apparent that the techniques employed have not allowed precise assessment of rate of glucose oxidation and the contradictory results which have been reported for the diabetic confirm this statement. Only if a proved steady state is established can an accurate measurement of this rate be obtained. To this end a labelled sugar load was given intraperitoneally in order to provide a labelled reserve of carbohydrate for tagged blood glucose maintenance. This technique was applied in a comparison of rates of glucose oxidation of diabetic and control rats.

Materials and Methods

The test animals were male albino rats whose average body weights for treatment groups ranged between 50 and 80 g. Fox cubes and water were offered ad libitum up to the time of sampling. Diabetes was established by the intraperitoneal injection of an aqueous solution of alloxan (200 mg/kg body weight) following a 40-hour starvation period. It was found that the chronically diabetic rat accumulated relatively large volumes of abdominal fluid. This unfavorable condition was corrected by maintaining the treated rats on insulin for 2 weeks, insulin being withdrawn 72 hours before use of the animal. Environmental temperature was controlled.

Different groups of diabetic and control rats were used for separate collections of blood glucose and expired CO_2 following the intraperitoneal injection of a labelled glucose load. The injection solutions, which were always of similar specific activity, consisted of either glucose-1-C^{14} or glucose-6-C^{14} made up in 5% unlabelled aqueous glucose. The injected dose consisted of 150 mg glucose; the total radioactivity administered was approximately 3×10^6 c.p.m. as measured in a windowless gas-flow counter. The specifically labelled glucose isotopes were obtained from Merck & Co. Ltd., Montreal. Blood glucose concentration and rate of expired CO_2 production were measured, as well as their specific activities after glucose-C^{14} administration.

Measurement of Specific Activity of Blood Glucose

Following injection of the labelled glucose load, different groups of three rats were individually bled at 15-minute intervals up to a period of 2 hours. A brief warming of the rat ensured the securing of 0.5 ml of blood from the tail vein. Total glucose content was determined by the method of Mendel *et al.* (8).

Interfering silver ions were removed from the deproteinized blood filtrate by precipitation as the chloride. Exactly 10 mg carrier glucose was added to appropriate aliquots of this filtrate and the phenylosazones were prepared. After the addition of recrystallized phenylhydrazine hydrochloride and sodium acetate to give respectively 1 *M* and 1.5 *M* concentrations, the solutions were heated in a boiling water bath for 2 hours, then allowed to cool slowly and to crystallize overnight at 5° C. The crystals were washed successively with distilled water and warm ethyl ether, then dissolved in hot alcohol.

An equal volume of warm water was added, and the solution was allowed to cool slowly to room temperature. Crystallization was allowed to continue overnight at 5° C. The crystals were transferred to tared aluminum planchets and radioactivity was measured in a gas-flow counter. Corrections for self-absorption were applied according to Karnovsky *et al.* (9). Phenylosazone melting points ranged from 205 to 212° C, indicating a satisfactory degree of purity.

Measurement of Specific Activity of Expired Carbon Dioxide

The respiration apparatus used was essentially that described by Gidez (10). Dry, CO_2-free air was drawn into the system by a vacuum pump at a rate controlled by a flowmeter and diffused through a fine mesh grid beneath a platform supporting the test animals. Silica gel prevented accumulation of moisture in the animal chamber. Two sets of interchangeable gas-washing bottles containing NaOH allowed the removal of samples without interruption of the gas flow. A terminal bottle containing $Ba(OH)_2$ indicated the efficiency of CO_2 absorption.

Immediately after receiving an intraperitoneal injection of radioactive glucose three rats were placed in the respiration chamber, and samples were removed at 30-minute intervals over a period of 5 hours. No samples were collected for the initial 30-minute interval.

Carbon dioxide was recovered as $BaCO_3$. Total carbonate values were obtained by titration or by a manometric technique, and these were in good agreement. Pads were prepared by filtration on filter paper, then transferred to plastic disks for counting. Corrections were made for self-absorption.

Results

It is apparent in Figs. 1 and 2 that the intraperitoneal injection of a labelled sugar load into both control and diabetic rats has maintained the specific activities of the blood glucose at constant levels for extended periods of time.

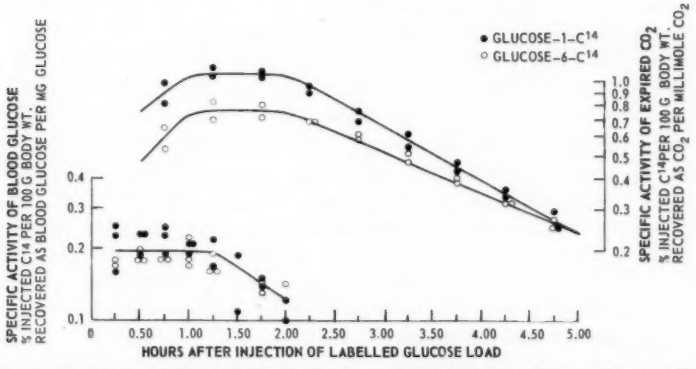

FIG. 1. Specific activities of blood glucose and expired CO_2 of control rats following injection of a labelled glucose load.

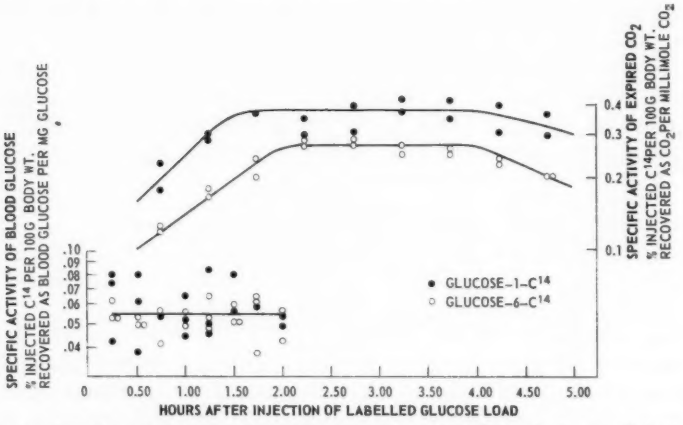

FIG. 2. Specific activities of blood glucose and expired CO_2 of alloxan diabetic rats following injection of a labelled glucose load.

Although the animals in each treatment group received 150 mg glucose by injection, the plateau in specific activity persisted during a longer interval in the diabetic than in the control. This effect is probably the result of a larger pool of body glucose, which is known to be characteristic of diabetes. There was no significant difference in the specific activities of the two isotopes, although the experiments for each isotope were conducted with different animals. The large difference in the heights of the plateaus for the two treatment groups is probably due to steady dilution of the plasma label by unlabelled glucose because of gluconeogenesis in the diabetic.

It may be noted in Table I that the 150-mg sugar load has had a variable effect on blood glucose concentration. When compared with non-injected rats for the same treatment group, the blood glucose levels of the injected control and diabetic rats have been raised in a large number of cases; and this variable effect is apparent throughout the 2-hour period.

Figs. 1 and 2 also show plateaus for the specific activities of the expired CO_2 for control and diabetic rats following the intraperitoneal injection of a labelled glucose load. Each graph shown is composed of data from two trials. As with the specific activity of the blood glucose, the plateau in the specific activity of the expired CO_2 for the diabetic rat was lower than and persisted longer than the plateau for the control. The maximum specific activity of the expired CO_2 appears at a considerably later time in the diabetic rat than in the control, presumably due to delayed equilibration of a larger glucose pool. The injection of the glucose-6-C^{14} has resulted in a longer delay than has the glucose-1-C^{14}. The specific activity values derived from glucose-1-C^{14} are higher than those from glucose-6-C^{14} for both treatment groups.

Table II illustrates that the CO_2 production rate for the diabetic rat was the same as that for the control rat.

Since plateaus have occurred in the specific activities of both blood glucose and expired CO_2 for both diabetic and control rats, it is apparent that a steady

TABLE I

Effect of 150-mg labelled glucose load on blood glucose level of rats

Time interval after injection (hr)	Control blood glucose (mg%)		Diabetic blood glucose (mg%)	
	Glucose-1-C^{14}	Glucose-6-C^{14}	Glucose-1-C^{14}	Glucose-6-C^{14}
0.25	162	152	516	884
	145	132	732	834
	135	152	484	636
0.50	172	142	358	884
	147	137	844	614
	137	122	234*	858
0.75	130	127	132*	360
	157	145	312	996
	140	147	488	888
1.00	137	117	484	920
	132	132	720	774
	—	132	784	666
1.25	145	142	980	644
	142	132	940	868
	137	137	980	800
1.50	145	120	566	902
	152	132	580	624
	125	120	260*	902
1.75	132	122	174*	940
	165	120	128*	650
	137	160	478	780
2.00	152	132	604	216*
	132	135	844	782
	132	137	710	888

NOTE: Three rats were sampled at each time interval.
Average blood glucose values for controls without a glucose load were 122.8 ± 6.9 mg% (S.D.). Average blood glucose values for diabetics without a glucose load were 527 ± 28.1 mg% (S.D.).
*These animals were not diabetic at time of sampling.

TABLE II

Expired CO_2 values of control and diabetic rats

Time interval after injection (hr)	Control		Diabetic	
	150 g total body wt.	154 g total body wt.	159 g total body wt.	163 g total body wt.
	μmoles CO_2/100 g/30 min		μmoles CO_2/100 g/30 min	
0.5–1.0	3087	3075	3530	3215
1.0–1.5	3332	4062	3649	3833
1.5–2.0	3508	3682	3240	3593
2.0–2.5	3805	3512	3439	3047
2.5–3.0	3229	3407	3649	4447
3.0–3.5	4043	3208	3240	4003
3.5–4.0	4098	4028	3683	3729
4.0–4.5	3056	3177	3299	3355
4.5–5.0	3403	4124	3754	4174
4.5 hr total	31561	32275	31483	33396

NOTE: Each column represents the production of three rats at successive time intervals.

state between the two components has been established for both treatment groups. It should be valid then to assume that blood glucose is being oxidized directly to CO_2 at constant rates in the control and diabetic rats while the

plateaus persist. With this assumption, the rates of oxidation of body glucose to CO_2 were calculated from the specific activity – time curves of plasma glucose and CO_2 thus: $(A \times B)/C$,

where A = specific activity of expired CO_2 at the plateau, expressed as % of the injected C^{14}/100 g body weight recovered as CO_2/mmole CO_2,

B = rate of CO_2 production, expressed as mmole CO_2/100 g/hr,

C = specific activity of blood glucose at the plateau, expressed as % of the injected C^{14}/100 g body weight recovered as blood glucose/mg glucose.

Table III shows that the rates of glucose oxidation and thus the percentage of CO_2 derived from glucose are somewhat higher for the diabetic animals than for the controls for both isotopes. It is apparent that oxidation is not

TABLE III

Glucose oxidation rates of control and diabetic rats

Treatment	Rate of glucose oxidation (mg/100 g/hr)		Glucose-1-C^{14} / Glucose-6-C^{14}	CO_2 derived from glucose (%)
	Glucose-1-C^{14}	Glucose-6-C^{14}		
Control	38.7	26.6	1.45	18.2
Diabetic	51.9	36.5	1.42	23.9

impaired by either oxidative pathway in the diabetic. However, since only single values are available, a statistical test for the significance of the apparent increases in oxidation rates in the diabetic cannot be made. But, because the ratios between the rates of oxidation for glucose-1-C^{14} and glucose-6-C^{14} are similar for both treatment groups, it is apparent that the diabetic lesion has not affected one oxidative pathway disproportionately. These values for the C_1/C_6 ratios are in good agreement with those of Stähelin *et al.* (11) for the normal guinea pig and those of Spiro and Ball (12) for the normal rat.

Discussion

The intraperitoneal injection of a labelled glucose load has resulted in persistent plateaus with respect to the specific activities of the blood glucose and the expired CO_2. Strisower and Searle (2) reported that the intravenous injection of a glucose load resulted in a plateau in the specific activity of the blood glucose as long as the blood sugar concentration remained elevated. Reichard *et al.* (13) confirmed this finding. It was concluded that hepatic glucose output is largely or totally suppressed during hyperglycaemia. On the contrary, Steele and Marks (14) showed an undiminished rate of dilution of labelled blood glucose during hyperglycaemia and they concluded that the output of unlabelled glucose by the liver continued during the period of hyperglycaemia. Since there was no consistent pattern of hyperglycaemia in the control rats in the current trials, this condition does not appear to have been the causative factor for the plateau.

To allow the formation of a plateau in the blood glucose specific activity, regulatory mechanisms for the release and utilization of glucose must be assumed. Both the liver and the intestinal mucosa (15) have been shown to be concerned with regulation of glucose release. But conflicting evidence has been presented concerning liver storage of glucose in the diabetic. Ashmore *et al.* (16) reported decreased formation of glycogen in diabetic rat liver while Barker and Sweet (17) reported that the administration of excess glucose to the diabetic dog resulted in the deposition of appreciable glycogen in liver. Haft and Miller (18) showed that the diabetic rat liver disposed of large quantities of hexose with no increase in incorporation of acetate-1-C^{14} into fatty acid.

Recycling of C^{14} is considered to be insignificant when the glucose curve approximates a straight line (3). In normal dogs the carbon of uniformly tagged glucose acts as if, upon entry into the cell, it is either converted promptly to CO_2 or else it is diverted to pathways of such length that it does not ever become CO_2 during the 5 hours of the experiment (19).

The interpretation of the C_1/C_6 ratios which have been presented is based on the widely accepted assumption that no difference will occur in the rate at which carbon 1 and carbon 6 are metabolized to CO_2 by the glycolytic cycle, whereas carbon 1 will be preferentially metabolized to CO_2 in the phosphogluconate pathway. This assumption seems reasonable for a relatively short time interval after the injection of the labelled glucose.

If this assumption is correct, then the demonstration of a steady state between blood glucose and expired CO_2 should allow a comparison to be made between the rates of oxidation of glucose by the two oxidative pathways. A steady state has been demonstrated in these trials, and it has been shown that the rate of glucose oxidation to CO_2 by either pathway has not been reduced in the diabetic rat when compared with control values. Also, the ratio between the two rates of oxidation is not different from that of the controls. Since no alteration occurred in the total CO_2 production in the diabetic rat, it is apparent that the proportion of CO_2 arising from glucose via both oxidative pathways is not reduced.

Wide variations have been reported for both normal and diabetic oxidation rates using the different techniques mentioned in the introduction (1–6). However, these data were not derived from animals in which the labelled glucose and expired CO_2 were in a steady state.

The findings are in agreement with those of Spiro and Ball (12), who found that glucose was metabolized by both the phosphogluconate and glycolytic pathways in the intact normal rat. They are in disagreement with the conclusions of Milstein (20), who found little evidence for the occurrence of a pathway other than glycolysis for the utilization of glucose in the intact rat.

The findings fail to support the conclusions of Siperstein and Fagan (21) and Stadie (22), who concluded that the observed defect in fatty acid synthesis in the diabetic liver is due to a lack of glucose oxidation via the hexose monophosphate shunt.

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A COMPARISON OF FREE ADRENAL CORTICAL STEROIDS IN THE BLOOD OF A HIBERNATING AND NON-HIBERNATING MAMMAL[1]

ARLISS DENYES AND ROBERT H. HORWOOD[2]

Abstract

White rats and hamsters were fasted and exposed to cold ($6 \pm 1°$ C) for various periods of time. The adrenal glands were weighed and adrenal cortical steroids were extracted from the blood for separation by paper chromatography.

The adrenal weight was not a reliable index of adrenal cortical activity as measured by the quantities of circulating hormones. Two steroid fractions were separated from rat and hamster blood. The fast component was identified as corticosterone and the slow component as an amorphous fraction.

The adrenal cortical response to cold is different in rats and hamsters. The hamster does not have the typical alarm reaction and only trace quantities of hormones are found in the hibernating animal. Within 6 hours of arousal the concentrations of circulating adrenal cortical hormones have increased to or beyond those of the control animals. The changes in circulating adrenal cortical hormones of hamsters exposed to cold for varying periods of time can be correlated with changes in their intermediary metabolism and ion balance.

Introduction

No one has been able to implicate a hormone in the essential mechanism of hibernation (1). Alterations in the cellular metabolism of cold-exposed rats and hamsters, however, do occur (2, 3). It is possible, although not yet proved, that hormones are cofactors in intermediary metabolism (4). Therefore, the circulating adrenal cortical hormones in rats and hamsters have been measured as an initial step toward the correlation of metabolic alterations and hormonal changes in hibernating and non-hibernating mammals.

Methods

Male white rats (Wistar strain) and male golden hamsters (*Mesocricetus a. auratus*) were kept at an ambient temperature of $20 \pm 2°$ C and in a cold room at $6 \pm 1°$ C. Purina laboratory chow and water were provided ad libitum and only the food was removed during the fasting experiments.

Rats to be kept in the cold for more than 1 week had their tails amputated as subadults 1 month before being placed in the cold room. The wound healed quickly and thus no problems of necrosis in the cold were encountered.

Blood was obtained by cardiac puncture after intraperitoneal Nembutal anaesthesia. With hibernating hamsters no anaesthetic was necessary and blood was taken from the cut aorta.

The free-circulating corticosteroids in the blood were extracted by the method of Bush (5). Known quantities of steroid were taken through the extraction process and each discarded fraction was chromatogrammed. None

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of the discarded fractions had measurable quantities of steroid. A 95 to 97% recovery of the known amount of steroid was achieved.

The extract was chromatogrammed according to the method of Bush (5). A single lot of Whatman No. 4 paper was washed in a benzene–methanol–water solvent which removed considerable fluorescent material. A benzene–methanol–water mixture (2:1:1) was equilibrated in a closed chamber at $35 \pm 0.5°$ C for 8 hours. The chromatogram was placed in the chamber without touching the solvent for 12 hours of equilibration at $35 \pm 0.5°$ C. Following this, sufficient benzene to cover the tab of the chromatogram was added to the chamber through a stoppered hole in the top. Separation of the cortical steroids was obtained at $35 \pm 0.5°$ C for 5 hours. In order to overcome the interference of impurities, the chromatograms were sprayed with 10% NaOH in 60% aqueous methanol and dried for 2 minutes at 70 to 75° C. After this treatment the steroids gave a brilliant yellow fluorescence in ultraviolet light.

The steroid concentration was determined by measuring the optical density of the chromatogram to ultraviolet light as described for amino acids by Block *et al.* (6). The chromatograms were measured within 24 hours of spraying with a photovolt densitometer equipped with an ultraviolet light source (254 mμ) and a $\frac{1}{4}$-inch aperture. The optical density was measured every $\frac{1}{4}$ inch beginning at the origin and moving toward the solvent front. An ultraviolet density curve was prepared by plotting the optical density against distance from the origin (Fig. 1). Two curves were drawn from each chromato-

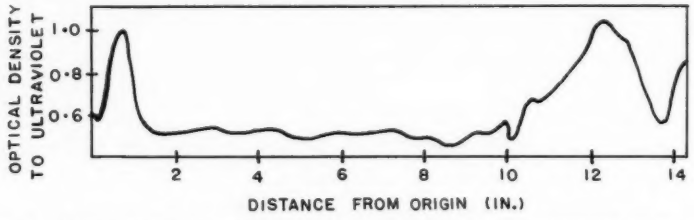

FIG. 1. A tracing of the area–density curve of a typical chromatogram under ultraviolet light.

gram and the area, minus the background, under each determined with a planimeter. The average area obtained from the duplicate curves was compared with a standard curve derived by plotting the areas of triplicate chromatograms of known amounts of cortisone against the quantities used. This relationship is shown by Fig. 2. A maximum deviation of 10% occurred in the area–density readings for this standard curve.

Bush (5) has described a 15% error for this technique of extraction and measurement. These present results are in agreement and only differences of 20% or greater will be considered significant.

The relative values for the adrenal cortical steroids in the blood were calculated as follows:

$$\frac{\text{the cortisone equivalent (standard curve)}}{\text{volume of blood}} \times 100.$$

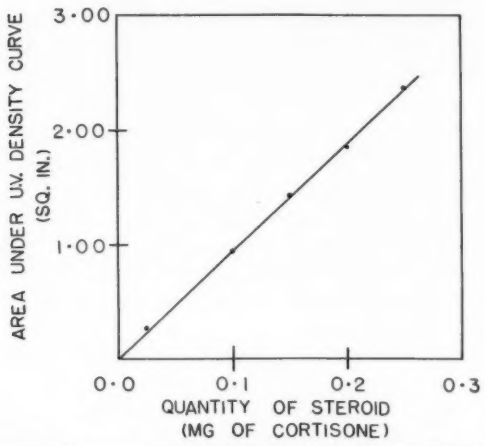

FIG. 2. The standard curve derived by plotting known amounts of cortisone against the areas of the ultraviolet density curves using the technique of Bush (5) and a photovolt densitometer.

The amorphous fraction was not identified and is expressed as (+) for the faint and (++) for a definite spot.

The steroids were identified on the basis of four criteria: the R_f values, yellow fluorescence with methanolic NaOH indicative of the Δ4,3-keto-configuration (5), the Zimmerman reaction (7) for the 17-ketone group, and the action of the α-ketol side chain with alkaline $AgNO_3$ (7).

The adrenal glands of each animal were removed and placed in chilled 0.9% saline. Fat and connective tissue were removed, the glands blotted on filter paper, and immediately weighed. The weights are expressed both as absolute values and as percentages of the total body weight.

The following groups of animals were studied:

Rats

1. Control-room animals feeding ad libitum.
2. Control-room animals fasted for 12 hours.
3. Animals exposed to cold for 48 hours and fasted for the last 12 hours in the cold (cold-stressed).
4. Animals living 2 and 4 months in the cold and feeding ad libitum.

Hamsters

1. Control-room animals feeding ad libitum.
2. Animals exposed to cold for 48 hours and feeding ad libitum (cold-stressed).
3. Animals in hibernation.
4. Animals aroused in the cold and feeding ad libitum.

Results

The adrenal weights of the hamsters (Table I) were not significantly different after prolonged cold exposure and during hibernation. The proportional

TABLE I

Body weight, adrenal weight, proportional adrenal weight, relative corticosterone value, and amorphous-fraction component of control hamsters and hamsters kept at 6±1° C for various periods of time

Group	No. of animals	Body weight (g)	Paired adrenal weight (mg)	Proportional adrenal weight (mg/100 g)	Cortico-sterone value	Amorphous fraction
Control	11	121±13*	25.3±3.5	21.0±1.9	1.70±0.32	+
48-hour cold-exposed	14	109±8	25.4±2.9	23.2±2.3	1.36±0.14†	−
Hibernating	7	95±11	21.0±5.7	22.3±2.3	trace to 0.38†	−
3-hour aroused	7	87±8	19.3±2.5	22.2±3.7	1.23±0.11†	+
6-hour aroused	7	90±9	18.7±3.1	21.1±3.3	1.77±0.63	++

* Standard deviation for small numbers.
† Significantly different from control.
− Material not detectable.
+ Fraction present but faint.
++ Fraction present as a distinct spot.

weight increased significantly ($P<0.01$) after 48 hours of cold exposure. This was due to a general loss in body weight not shared by the adrenal glands. In contrast to this, the absolute adrenal weights of the control and cold-stressed animals were similar.

The white rats had a greater variation in adrenal weight than the hamsters (Table II). The rats exposed to cold for 2 and 4 months had significantly

TABLE II

Body weight, adrenal weight, proportional adrenal weight, relative corticosterone value, and amorphous-fraction component of control rats, fasted rats, and rats both fasted and non-fasted in the cold (6±1° C)

Group	No. of animals	Body weight (g)	Paired adrenal weight (mg)	Proportional adrenal weight (mg/100 g)	Cortico-sterone value	Amorphous fraction
Non-fasted control	7	500±42*	46.3±7.5	9.4±2.0	1.35±0.01	+
12-hour fasted control	7	514±59	43.9±4.6	8.6±0.8	1.63±0.24†	+
48-hour cold-exposed, 12-hour fasted	7	418±72	54.0±14.3	13.4±4.7	1.76±0.41†	−
Non-fasted, 2-month cold-exposed	7	400±30	62.3±6.6	15.9±2.1	1.24±0.07	++
Non-fasted, 4-month cold-exposed	7	339±66	53.4±7.8	16.3±3.8	1.70±0.29†	−

* Standard deviation for small numbers.
† Significantly different from non-fasted control.
− Material not detectable.
+ Fraction present but faint.
++ Fraction present as a distinct spot.

greater proportional adrenal weights ($P<0.01$) than the control animals. Control animals fasted for 12 hours had a slight decline in proportional adrenal weight. The cold-stressed group had significantly heavier adrenals than the fasted control animals ($P<0.01$), but did not differ significantly from the non-

fasted control animals ($P=0.08$). The proportional adrenal weights did not change in direct relation with the absolute weights among the various groups of rats. In the 2- and 4-months cold groups the absolute adrenal weight decreased with prolonged cold exposure while the proportional weight increased due to a rapid loss of body weight after 2 months of cold exposure.

The blood of hamsters and of rats was found to contain a steroid with an average R_f value of 0.86. This material fluoresced with methanolic NaOH, gave a negative reaction to the Zimmerman test, and reduced alkaline $AgNO_3$. Similar results are obtained with corticosterone (5, 7). Zenker and Bernstein (8), using fluorometric technique, also identified the common adrenal cortical steroid of rat blood as corticosterone. Figure 3 represents some typical

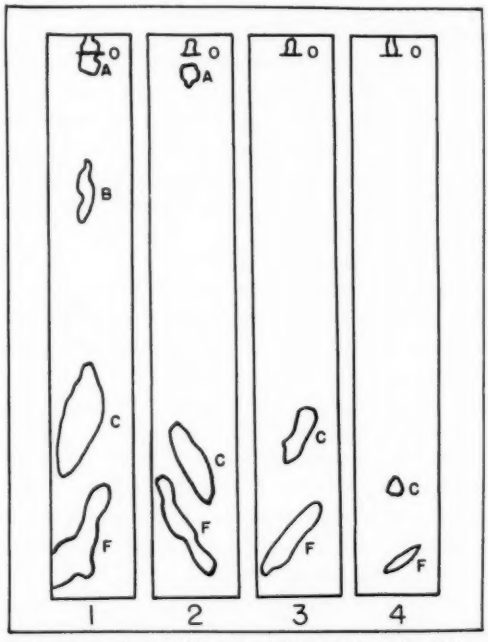

FIG. 3. Chromatograms of rat and hamster blood: (O) origin, (A) amorphous fraction, (B) cortisone standard, (C) corticosterone, (F) solvent front; (1) from rats exposed to cold ($6\pm1°$ C) for 48 hours and fasted for 12 hours, (2) from rats exposed to cold for 2 months, (3) from hamsters exposed to cold for 48 hours, (4) from hamsters in hibernation.

chromatograms from four of the groups of rats and hamsters. On certain of the chromatograms a second spot appeared close to the origin. This material fluoresced with methanolic NaOH, reduced $AgNO_3$, but failed to react with the Zimmerman reagent. A low R_f of 0.05, characteristic of components of the amorphous fraction of adrenal gland extracts, was obtained. For present purposes this material is designated, therefore, as an amorphous fraction component.

There was a 20% reduction ($P<0.05$) of the blood corticosterone of hamsters exposed to the cold for 48 hours as compared to the control value. No amorphous-fraction component was detected in these cold-stressed animals. Blood from hibernating hamsters had no detectable amorphous-fraction component and the corticosterone concentration was 77% less than that of the control animals. Hamsters aroused from hibernation for 3 hours had 69% more circulating corticosterone than the hibernating animals. Hamsters aroused from hibernation for 6 hours had variable corticosterone concentrations but the average value was similar to that of the control animals. These animals also had a marked amorphous fraction spot.

There was a 20% increase in blood corticosterone of rats that were fasted for 12 hours but not exposed to cold ($P<0.02$). When the rats were exposed to cold and also fasted the blood corticosterone remained significantly greater than the non-fasted control value. The rats exposed to cold for 2 months had particularly uniform blood corticosterone concentrations which were lower than any other group but not significantly different from that of the non-fasted control animals. There was a definite increase in the amorphous-fraction component in the blood of rats exposed to the cold for 2 months. After 4 months of cold exposure, the amorphous fraction component was not detectable in the rat blood and the corticosterone concentration was as high as that of the fasted, cold-stressed animals.

Abrupt exposure to cold accompanied by fasting caused a significant increase in the corticosterone concentration of rat blood. Hamsters exposed to cold for a few days, but not fasted, had a significant decline in blood corticosterone as compared to their control value. Thus, fasting alone causes an increase in the blood corticosterone in rats and this effect may be slightly augmented by cold exposure, while in hamsters cold exposure alone causes a significant decline in blood corticosterone.

Fasting had no effect on the presence of the amorphous-fraction component in the blood of control rats. This fraction is detectable neither in the blood of cold-stressed rats nor cold-stressed hamsters. The greatest quantity of amorphous-fraction component was found in the blood of hamsters aroused for 6 hours and in non-fasted rats exposed to cold for 2 months. These two groups are comparable in terms of the number of weeks spent in the cold. Therefore, alterations in the amorphous-fraction component are similar in rats and hamsters.

The two steroid fractions in rat, but not in hamster blood, vary independently. This suggests that in the rat each one has its own release mechanism.

The blood corticosterone concentration cannot be predicted from the proportional adrenal weights. For example, there was no significant change in the proportional adrenal weight of cold-stressed hamsters as compared to control animals but the blood corticosterone value was lower than that of the control animals. Similarly, fasted control rats had a slight decline in gland weight and a higher blood corticosterone value than non-fasted control rats. With the fasted, cold-stressed rats there was a gain both in adrenal weight and in corticosterone value.

Discussion

The adrenal hypertrophy found in the cold-exposed rats has been reported previously by Emery *et al.* (9), Katsh *et al.* (10), Deane and Lyman (11), and Héroux and Schonbaum (12). The gain in weight of the gland has been attributed to an increase in the width of the cortical zona fasciculata in which the bulk of the steroid production is thought to occur. Similarly, the changes in adrenal weight in the hamster are in agreement with those reported by Deane and Lyman (11). It is typical of the cold-exposed hamster to exhibit no enlargement of the adrenal gland nor major histological changes on initial and prolonged exposure to cold.

These observations on gland size would suggest a high adrenal cortical steroid level in the cold-exposed rat and a lower level in the cold-exposed hamster. This hypothesis presupposes that hypertrophy implies hyperactivity. These present results do not support this hypothesis. The weight of the adrenal gland is not a reliable index of the circulating level of adrenal cortical hormones in cold-exposed rats and hamsters.

The blood corticosterone concentrations of hamsters are in agreement with the conclusions of Deane and Lyman (based on histochemical techniques) only inasmuch as the control and acclimated animals present a similar picture. In the 48-hour cold-exposed and hibernating hamsters there is a significant decrease in the corticosterone level which is not indicated by the histochemical techniques. The very low corticosterone concentration of the hibernating hamster is not unexpected in view of the low metabolic rate of the tissues at that time. The rapid increase of both the corticosterone and the amorphous-fraction component during arousal is in keeping with the remarkable increase in metabolic rate characteristic of the arousing hamster. Within 3 hours the corticosterone concentration of the cold-stressed hamster is achieved, and by 6 hours a concentration similar to that of a control animal is reached. This return to normal blood steroid levels could be accomplished by the storage of steroid just previous to hibernation followed by immediate release or by a high rate of production during the arousal period. Suomalainen (13) demonstrated a storage of materials in the hypophysis of the hibernating hamster but Deane and Lyman (11) did not find an accumulation of products in the adrenals of such animals. It would appear, therefore, that the arousing hamster synthesizes adrenal cortical steroids at a rapid rate under the influence of the pituitary, and thus achieves a normal blood concentration within 6 hours.

The production of amorphous-fraction component in hamsters aroused for 6 hours is excessive as compared to the other groups. A potent mineralocorticoid has been demonstrated in rat blood at the R_f value expected from the amorphous fraction (14). Aldosterone, an amorphous-fraction component, greatly influences the plasma and urine electrolyte concentrations by promoting the retention of Na^+ (15, 16). According to Denyes and Hassett (2) the urinary K^+ of the acclimated hamster is twice that of the Na^+ concentration. The cold-stressed hamster, in which no amorphous fraction was detected, excreted less K^+ and more Na^+ in the urine. This relation between low amorphous-fraction component and loss of Na^+ is found in the hibernating

hamster where a large amount of Na^+ accumulates in the bladder. In hibernation the loss of both ions is very high and the effect of low temperature on the cell membrane has to be considered. On the whole, however, the pattern of Na^+ and K^+ excretion and the amount of amorphous-fraction component in the blood of hamsters in the cold appear to be directly related.

Both corticosterone and aldosterone stimulate glycogenesis (17). The average total carbohydrate in the liver of the control hamsters is 8.2%, of cold-stressed hamsters 2.2%, of 3-week acclimated hamsters 2.6%, of hibernating hamsters 2.7%, and of 6-hour aroused hamsters 5% (18). During the early period of arousal, this stored carbohydrate is used (19). The low level of steroid in the hibernating hamster would encourage the glycogenolysis during early arousal, whereas the high level of steroid in the 6-hour aroused animal would stimulate glycogenesis. The low concentration of the steroids in the cold-stressed hamster may be one factor which influences the drastic depression in hepatic glycogenesis.

The changes in blood corticosterone concentration of the rats exposed to cold for varying periods of time are described by the general adaptation syndrome of Selye (20). The "alarm reaction" is not evident in the hamster. The adrenal cortical steroid concentration fell below that of the control hamsters rather than increasing as in the rat. If the 6-hour aroused hamsters can be considered as acclimated animals, then this group also does not fit Selye's description of the typical "stage of resistance". These acclimated hamsters had steroid levels as high as those of the control animals.

Regardless of these differences in adrenal cortical response to cold both the rat and the hamster maintain a thermogenesis adequate for survival in the cold for many months. According to the biochemical feedback hypothesis of Potter (21) a change in a metabolite or hormone concentration could affect the production and nature of the cellular enzymes. This would provide true enzymatic adaptation to new conditions. Evidence of this kind of adaptation is shown in the work of Masoro and co-workers on cold-exposed white rats and is summarized in a recent publication (4). This adaptation occurs during the initial stages of cold exposure in the white rat. The adrenal cortical hormones are capable of participating in enzymatic adaptation (21, 22). Therefore, the dissimilar adrenal response of the rat and hamster during cold exposure suggests that each species develops different enzymatic relations in the cold. Although hibernation is a complex process, it is possible that the lack of adrenal cortical "alarm reaction" and the consequent difference in the nature and timing of the general metabolic alterations may be a reflection of the ability of the one species to hibernate.

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CATALASE LEVELS AND RADIOSENSITIVITY OF LIVER CELLS[1]

D. K. MYERS

Abstract

Following exposure of regenerating liver to 580 r X radiation at a time when the average amount of DNA per cell nucleus has reached 1.6 times the normal value, the usual increase in number of cell nuclei was retarded while the average amount of DNA per cell nucleus continued to rise until it approached twice the normal value. Cell division was delayed for an unusually long period following irradiation at this stage of development. On the other hand, there was no corresponding increase during liver regeneration in the effects of high doses of X radiation on diphosphopyridine nucleotide concentrations in the liver. Although the liver catalase concentration decreased during liver regeneration, inhibition of catalase activity failed to increase the radiosensitivity of liver cells.

Introduction

Irradiation of normal rat liver has been found to delay the subsequent accumulation of deoxyribonucleic acid and increase in cell number which occurs in the liver of control animals following partial hepatectomy (1–3). The effect of irradiation of regenerating liver at different stages of growth was studied in the present investigation. As will be shown below, the proliferation of liver cells became more sensitive to delay by X irradiation shortly before rapid cell division would normally occur.

One explanation for this increase in radiosensitivity might be provided by the observed decrease in catalase concentrations in growing liver cells (4). Interest in this possibility was stimulated by the report (5) that catalase does protect the glycolysis of dilute tumor cell suspensions against inhibition by high doses of X rays; it was suggested that X radiation acted selectively on tumor cells because these cells contain less catalase than normal cells (5). The inhibition of glycolysis following irradiation appears to be due to destruction of diphosphopyridine nucleotide (6, 7). In the present investigation, therefore, the effects of catalase inhibition have been studied on two aspects of the radiosensitivity of normal liver cells, i.e. (*a*) on the delay in cell division following exposure to low doses of radiation, and (*b*) on the loss of diphosphopyridine nucleotide from the cells following exposure to high doses of radiation.

Methods

In order to study the effects of X radiation on liver regeneration, 10-week-old female rats, weighing approximately 180 g, were exposed to local irradiation over the liver region, either before or after $68 \pm 3\%$ of the liver had been removed from these animals by a partial hepatectomy as described previously (3). During irradiation, the conscious animal was restrained in a rectangular plastic chamber with internal dimensions of $3.8 \times 4.4 \times 20$ cm; the major portion of the animal was protected by lead shielding and only the liver region

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was exposed, through a hole 3.6 cm in diameter, to X rays from a 2000-kvp source operated at 1.5 ma (3). The dose rate, measured in air, was 300 r/minute. Under the above conditions, the usual dose employed in these experiments (580 r) represented approximately 37% of the dose which would produce 50% mortality in non-hepatectomized animals within 30 days. All of the animals exposed to 580 r over the liver region appeared to remain in good health.

After partial hepatectomy, each rat was kept in an individual cage in a ventilated room which was held at 24–27° C and lighted for 12 hours each day from 6 a.m. to 6 p.m. All animals were allowed food and water ad libitum and were ultimately killed for liver analyses at approximately the same time of day, i.e., between 9 a.m. and 12 a.m. Samples of regenerating liver from these animals were excised immediately after death and homogenized in an ice-cold solution containing 0.14 *M* NaCl plus 0.003 *M* $CaCl_2$. The liver homogenate was then analyzed for protein by the biuret reaction and for deoxyribonucleic acid by a diphenylamine reaction on hot trichloroacetic acid extracts; the number of cell nuclei in samples of the same homogenate was counted in a haemocytometer. Details of these procedures are described in a previous publication (3).

The catalase activity of aqueous liver homogenates was measured by the method of Feinstein (8), in which sodium perborate serves as the source of hydrogen peroxide. A small correction for the catalase activity of blood present in the liver homogenates was made on the basis of the relative haemoglobin concentrations in blood and liver; this correction amounted to approximately 1% of the total catalase activity of normal liver and consequently did not alter the results appreciably. The catalase activity of the liver was inhibited by intraperitoneal injection of a 5% (w/v) solution of 3-amino-1,2,4-triazole (Agricultural Chemicals Division, American Cyanamide Co., New York) into the animals (9).

In experiments on the diphosphopyridine nucleotide (DPN) content of various tissues, 7- to 10-week-old animals were exposed to whole body irradiation at a dose rate of 1600 r/minute and the tissues were excised for analysis between 5 and 30 minutes after irradiation. The tissue sample was ground with 5% (w/v) trichloroacetic acid within a few seconds after removal from an anaesthetized animal (10). After centrifuging of the tissue suspension and neutralizing of the supernatant fluid, the amount of DPN in this extract was measured by an enzymic method using ethanol and purified alcohol dehydrogenase (Nutritional Biochemicals Corp., Cleveland) with 0.03 *M* sodium pyrophosphate as buffer (10).

Results

Liver Regeneration after Irradiation at Different Intervals

The results obtained when the regenerating liver was exposed to X radiation 24 hours after partial hepatectomy are shown in Fig. 1. At this time, cell division has not commenced and, in our animals, DNA accumulation was still low; presumably a large proportion of the cells had not yet started to synthe-

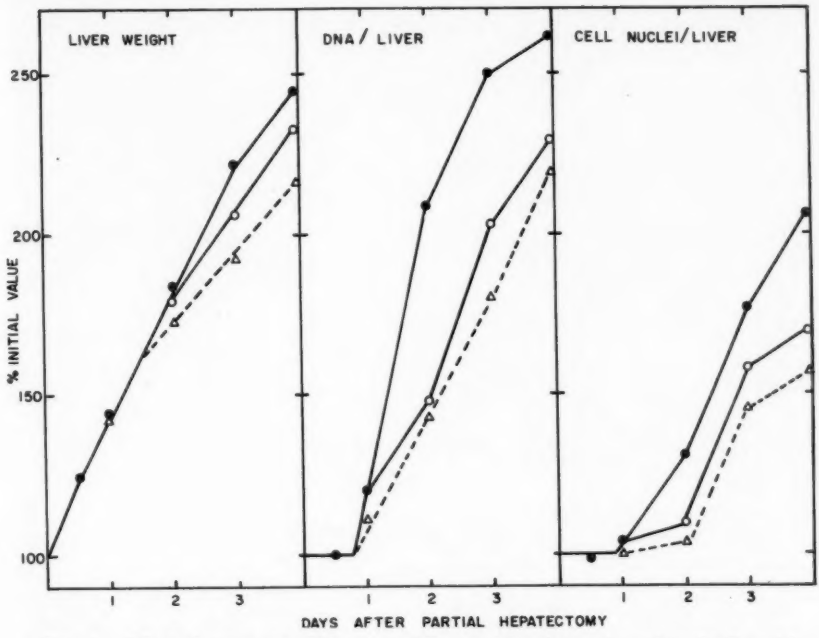

FIG. 1. Effect of exposure to 580 r on total liver weight, the total number of cell nuclei, and total amount of DNA in the regenerating liver. Each point represents the average results obtained from six or more rats: control animals (●); irradiated 1 day after partial hepatectomy (○); irradiated just before partial hepatectomy (△).

size DNA. Irradiation with 580 r delayed DNA accumulation and cell division for approximately 1 day (Fig. 1); the effects are similar to those observed when the normal liver was irradiated just before partial hepatectomy (3).

Different results were observed when the regenerating liver was exposed to radiation 48 hours after partial hepatectomy (Fig. 2). At this time, the average amount of DNA per cell nucleus had reached a maximum at 1.6 times the normal level (Fig. 3). Assuming that the amount of DNA in a given cell nucleus reaches twice the normal level before cell division occurs (11), it would appear that a large proportion of the liver cells were nearing the point of cell division at the time of irradiation. Irradiation with 580 r prevented the normal increase in number of cell nuclei during the next 24 hours (Fig. 2); in fact, the total number of cell nuclei in the liver did not increase rapidly during the first 5 days after the irradiation. However, the irradiation had no immediate effect on DNA synthesis (Fig. 2). The average amount of DNA per cell nucleus continued to rise until it reached approximately double the normal level and then remained at this value until cell division recommenced (Fig. 3). These results resemble those obtained after irradiation of growing cells in tissue culture with 1000 r (12).

Since cellular proliferation in the regenerating liver appeared to be exceptionally sensitive to irradiation at this stage of growth, the effect of lower

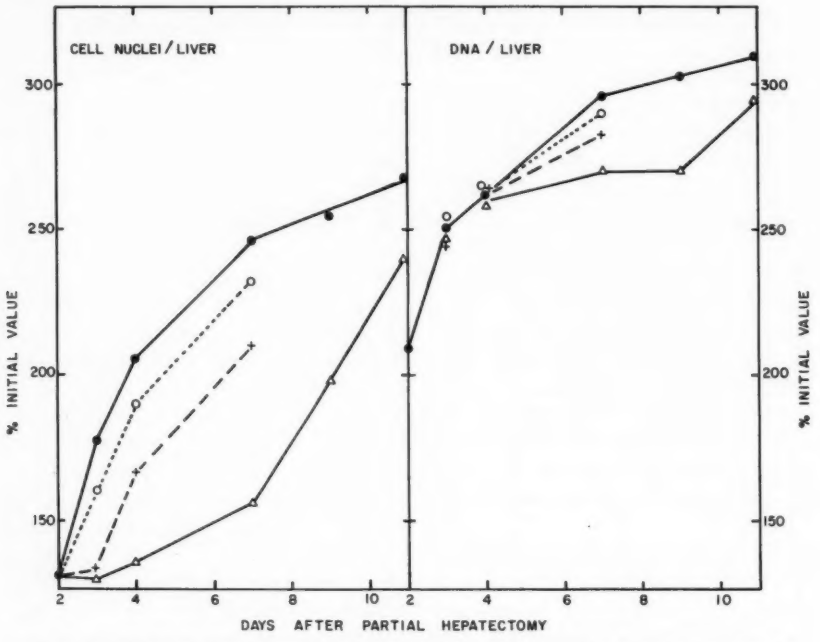

FIG. 2. Effect of irradiation of liver region 2 days after partial hepatectomy on the total number of cell nuclei and total amount of DNA in the regenerating liver. Each point represents the average results obtained with six rats: control animals (●); exposed to 50 r (○); exposed to 170 r (+); exposed to 580 r (△).

doses of radiation was studied. Irradiation with 170 r still produced an appreciable delay in cell division (Fig. 2). A small effect was also observed after exposure to 50 r (Fig. 2) but the differences in the average numbers of cell nuclei in the livers of the control and irradiated animals were not statistically significant.

Liver weights and total protein contents were also determined in all of the experiments illustrated in Figs. 1 and 2. As in previous experiments (3), irradiation had no immediate effect on the increases in liver weight and total protein during liver regeneration.

The marked effect of irradiation shown in Fig. 2 cannot be ascribed to stimulation of adrenal secretions in the irradiated animals. A similar delay in cell division was observed following irradiation of the regenerating liver in adrenalectomized animals (Table I).

Catalase Levels

In confirmation of the results of Stein *et al.* (4), it was found that the concentration of catalase in regenerating liver 48 hours after partial hepatectomy had decreased to approximately half the normal value (Table II). When expressed on the basis of catalase activity per cell nucleus, a similar decrease is observed in the livers of starved animals (Table II).

TABLE I

Effect of X irradiation on liver regeneration in control and adrenalectomized rats

Average results are given, together with the standard deviation of the individual measurements, followed by the number of animals in parentheses. The effect of irradiation on increase in liver weight is not statistically significant, while the effect of irradiation on increase in number of cell nuclei/liver is statistically significant ($p < 0.05$) in both the control and adrenalectomized animals

	Days after partial hepatectomy	Control rats		Adrenalectomized rats	
		Non-irradiated	580 r X rays on day 2	Non-irradiated	580 r X rays on day 2
Liver weight (g)	2	4.4±0.5 (18)		4.3±0.2 (3)	
Liver weight (g)	7	6.8±0.5 (16)	6.7±0.3 (6)	7.9±0.2 (3)	8.2±0.5 (3)
Increase in liver weight (g)	—	2.4±0.5	2.3±0.5	3.6±0.2	3.9±0.4
10^8 cell nuclei/liver	2	8.2±0.6 (18)		8.5±0.5 (3)	
10^8 cell nuclei/liver	7	15.1±0.6 (16)	9.7±0.3 (6)	13.8±0.5 (3)	10.0±1.6 (3)
Increase in number of cell nuclei × 10^8	—	6.9±0.6	1.5±0.6	5.3±0.6	1.5±1.4

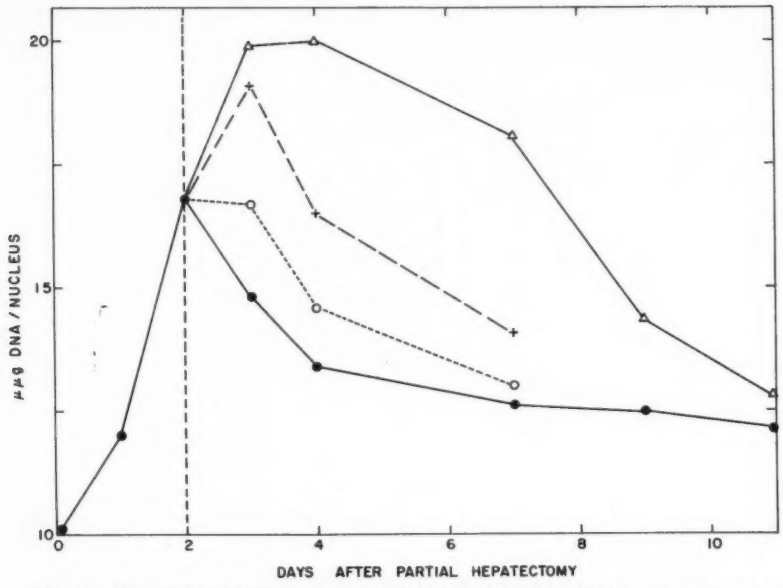

FIG. 3. Changes in the average amounts of DNA/cell nucleus in the regenerating liver following irradiation on the second day after partial hepatectomy: control animals (●); exposed to 50 r (○); exposed to 170 r (+); exposed to 580 r (△).

TABLE II

Catalase activity of rat liver under various conditions

Treatment of animals	Number of animals	Catalase activity (meq perborate/5 min)	
		Per g liver	Per 10^8 cell nuclei
Controls	9	780	320
24 hr after partial hepatectomy	4	527	268
48 hr after partial hepatectomy	4	336	195
24 hr fasting	2	640	179
48 hr fasting	2	520	134
1 hr after 1 g AT/kg	3	39	15
17 hr after 1 g AT/kg	4	196	80
17 hr after 2 g AT/kg	4	150	61

In order to determine the effect of low catalase levels on the radiosensitivity of the liver cells, liver catalase was inhibited by injection of 3-amino-1,2,4-triazole (AT) into the rats before irradiation (9, 13); 95% inhibition was observed 1 hour after the intraperitoneal injection of 1 g AT/kg (Table II). According to Thomson and Klippel (14), the catalase activities of each of the subcellular fractions of the liver cells should be inhibited to approximately the same extent. However, the liver would still contain appreciable amounts of free AT 1 hour after the injection (15) and the presence of this compound may provide slight protection against the effects of radiation (16). Since

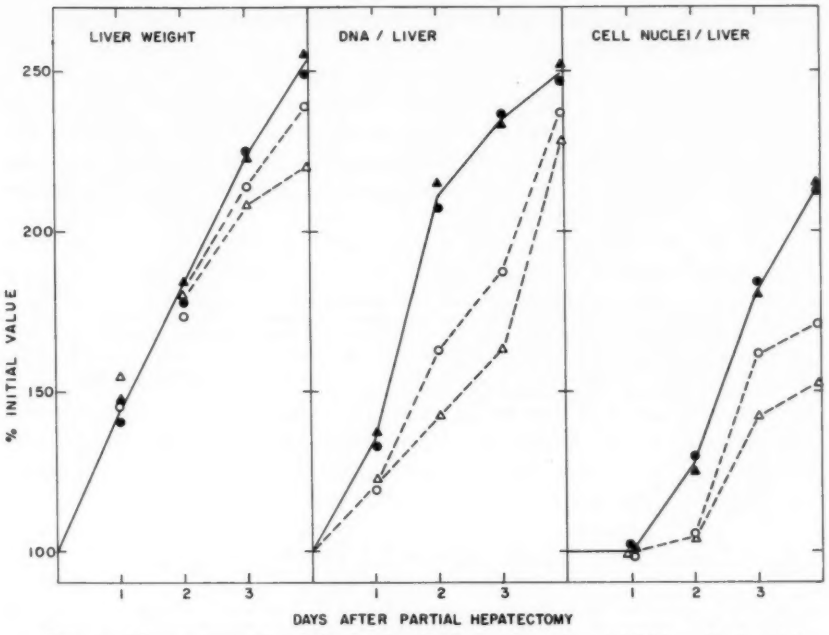

FIG. 4. Liver regeneration following irradiation and catalase inhibition. Each point represents the average values obtained from four rats:
▲ 1 g AT/kg at minus 3 hours; △ same, plus 580 r at minus 2 hours. ● 2 g AT/kg at minus 19 hours; ○ same, plus 580 r at minus 2 hours.

most of the free AT is eliminated from the animal body within the first day after the injection (13), a second series of experiments was carried out 17 hours after the injection of 2 g AT/kg. Under these conditions, the liver catalase activity was still reduced to 20% of its normal value (Table II).

Liver Regeneration after Irradiation and Catalase Inhibition

The rate of liver regeneration was not significantly altered following injection of AT (cf. Fig. 1 and Fig. 4). The effect of exposure to 580 r prior to partial hepatectomy (Fig. 1) was decreased slightly when the animals were injected with 1 g AT/kg 1 hour before irradiation (Fig. 4). Apparently the radiosensitivity of the liver cells had not been increased appreciably by 95% inhibition of the catalase activity. In the second series of experiments, injection of 2 g AT/kg 17 hours before irradiation did not appear to alter the radiosensitivity of the liver cells significantly (Fig. 4), even though the catalase activity of the liver was still 80% inhibited (Table II).

DPN Concentrations after Irradiation and Catalase Inhibition

After exposing intact animals to high doses of radiation, DPN concentrations in the liver, spleen, and thymus were reduced (Table III); the percentage loss of DPN was greatest in thymus, slightly less in spleen, and least in the liver. The catalase activity of these three tissues decreases in the reverse

order (17, 18), suggesting that a high catalase activity might protect the tissues against loss of DPN during irradiation. However, regenerating liver, examined 2 days after partial hepatectomy, did not exhibit greater loss of DPN than normal liver after irradiation, even though the regenerating liver contains less catalase than normal liver. Furthermore, the loss of DPN from the tissues was not augmented by injection of 1 g AT/kg into the animals (Table III) in order to inhibit liver and spleen catalase activities (13) before irradiation.

TABLE III

Effect of irradiation on DPN concentrations in rat tissues
The average values for DPN concentrations are given, together with the standard deviation of the individual measurements, followed by the number of animals in parentheses. A single asterisk indicates that the effect of irradiation on DPN concentration is statistically significant ($p < 0.05$), while a double asterisk indicates that the effect is highly significant ($p < 0.01$).

Tissue	Dose of radiation (kr)	DPN concentration (μmole/100 g tissue)	
		Control rats	AT-injected rats
Liver	0	77 ± 8 (13)	77 ± 6 (3)
	1.5	76 ± 2 (5)	—
	8	68 ± 9 (5)*	65; 72 (2)
	24	62 ± 4 (5)**	59 ± 7 (3)*
Regenerating liver	0	71 ± 10 (6)	—
	8	66 ± 6 (3)	—
	24	58 ± 3 (3)*	—
Spleen	0	33 ± 3 (10)	30 ± 1 (4)
	1.5	27 ± 1 (4)**	—
	8	18 ± 2 (6)**	17; 20 (2)**
	24	12 ± 1 (4)**	10 ± 1 (3)**
Thymus	0	24 ± 2 (4)	20; 23 (2)
	1.5	20 ± 1 (4)**	—
	8	10 ± 2 (3)**	10; 11 (2)**
	24	6 ± 2 (3)**	4; 4 (2)**
Blood	0	12 ± 1 (4)	12 ± 1 (4)
	8	12 ± 1 (3)	12 ± 1 (4)
	24	11 ± 1 (5)	11; 12 (2)

Discussion

The results of our experiments on DNA content of regenerating liver agree with previous observations concerning the effects of irradiation on the incorporation of labelled precursors into the DNA of regenerating liver (1, 2, 19), provided that differences in the rate of DNA synthesis in the livers of non-irradiated animals are taken into consideration. A low dose of X radiation (580 r) delayed the onset of rapid DNA synthesis in cells which had not yet entered this phase of development (Fig. 1). On the other hand, the same dose of radiation did not seem to interfere immediately with active DNA synthesis which was in progress at the time of irradiation, although cell division was still delayed under these conditions (Fig. 2). Consequently the average amount of DNA per cell nucleus following irradiation may be either lower (3) or higher (Fig. 3) than the corresponding values in non-irradiated regenerating liver,

depending on whether irradiation occurs prior to or during the phase of rapid DNA synthesis. It might be noted that the average amount of DNA per cell nucleus did not rise above twice the normal level in any of these experiments (cf. Fig. 3). The average wet weight of liver per cell nucleus also reached a maximum at less than twice the normal value for non-hepatectomized animals; on this basis, it would appear that the irradiated regenerating liver did not contain a large proportion of giant cells.

Exposure to 170 r on the second day after partial hepatectomy (Fig. 2), when the average amount of DNA per cell nucleus was high, delayed division for approximately the same length of time as exposure to 580 r 1 day earlier (Fig. 1), before most of the cells enter the phase of DNA synthesis. An explanation for this increase in radiosensitivity of cell division 2 days after partial hepatectomy is not yet available. The results of our experiments (Fig. 4) with a catalase inhibitor (AT) indicated that the increase cannot be ascribed simply to low catalase levels. At least two other enzymes that could act on hydrogen peroxide produced during irradiation might also be excluded from consideration. Peroxidase is present only in small amounts, if at all, in liver (21) while tryptophane pyrollase appears to be inhibited by AT in much the same manner as catalase (22).

The results of further experiments with AT indicated that the loss of DPN from various rat tissues following high doses of radiation (Table III) was not dependent on the catalase content of these tissues. This conclusion seems to disagree with the results obtained following irradiation of dilute cell suspensions in vitro (5); however, most of the effects observed in the latter experiments were probably due to hydrogen peroxide formed in the suspending medium (5, 7) rather than to products of irradiation formed within the cells. Further experiments are required to provide an explanation for the effects of irradiation on DPN in the tissues.

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NOTES

A HYPOCHOLESTEROLEMIC FACTOR IN MARINE STEROLS

E. REINER,[1] D. R. IDLER,[1] AND J. D. WOOD[2]

During recent years, several previously undescribed sterols have been isolated from marine invertebrates in this laboratory. 24-Methylenecholesterol was identified as the principal sterol of the butter clam (*Saxidomus giganteus*) (1) and a major component of several other edible molluscs (2). Other sterols of the clam have not been identified but evidence has been presented indicating that cholesterol is not a significant component (1, 2).

We wish to report that a sterol mixture of the butter clam exerts a hypocholesterolemic effect in chicks.

Previously, such effects have been observed following controlled feeding of cholestanol (3), β-sitosterol, ergosterol, or stigmasterol (4), and from a substance found in lingcod liver oil (5, 6).

The mixture of clam sterols was isolated according to published procedures (1, 2). The sterols were purified with charcoal and one recrystallization from ethanol.

Groups of sixteen 8-day-old chicks were maintained on either a standard diet (5), a standard diet supplemented with 1% cholesterol, or a standard diet supplemented with 1% cholesterol and 1% clam sterol mixture.

On the 14th day of test feeding, blood samples (1.25 ml) were taken from the brachial vein and pooled in lots of four prior to collection of serum. Serum cholesterol levels were measured according to the method of Sperry and Webb (7). The results are summarized in Table I.

TABLE I

Effect of diet on serum cholesterol levels

	Basic diet	Basic + 1% cholesterol	Basic + 1% cholesterol + 1% clam sterol
	Serum cholesterol* (mg%)		
	135	585	262
	192	707	354
	109	581	301
	191	638	507
Mean ± S.E.	157 ± 21	628 ± 30	356 ± 54
Food consumption†	17.7	17.0	17.1
Weight gain‡	95.4	89.1	88.1

* Analysis of variance: groups 2 and 3, $P = 0.01$–0.001; groups 1 and 2, $P = <0.001$; groups 1 and 3, $P = <0.001$.
†Grams/chick/day.
‡Grams/chick.

Feeding experiments, using synthetic 24-methylenecholesterol (8) will be the subject of a future communication.

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A NOTE ON THE PAPER CHROMATOGRAPHY OF SOME INDOLE ACIDS AND THEIR DERIVATIVES

R. A. HEACOCK AND M. E. MAHON

A very widespread interest has developed in the paper chromatography of indole derivatives during the last decade, stimulated by the efforts of several groups of workers: firstly, groups interested in the analysis of the urinary indoles found in many pathological conditions; secondly, groups studying the occurrence and metabolism of many indolic plant growth substances; and thirdly, workers interested in the chemistry of several physiologically important alkaloids containing the indole nucleus (for lists of references see ref. 1, p. 114, 1 and 2, p. 313).

In these laboratories we have been primarily concerned with a study of the paper chromatographic behavior of the 5,6-dihydroxyindoles (3), and in view of the success obtained using one of the Zaffaroni type systems (i.e. formamide-treated paper with a non-polar running solvent) (cf. 4) with this group of compounds it was decided to investigate the behavior of several other indole derivatives in this type of system. Systems using formamide-treated papers have been widely used for some years in chromatographic studies on the ergot alkaloids (2, p. 359; 5; 6). During the course of our investigations the authors became aware of a publication of Procházka, Šanda, and Macek (7) on the paper chromatography of indole derivatives, which reported the R_f's of several indoles on formamide-treated paper with formamide-saturated cyclohexane, benzene, and chloroform as running solvents. No R_f values of any indole acids (or acid derivatives) were reported when either cyclohexane or benzene was the mobile phase and only a few were given when chloroform was the mobile phase; in this case the R_f values tended to be rather low (e.g. those of 3-indolecarboxylic acid, 3-indoleacetic acid, 3-indole-γ-butyric acid, and 3-indolealdehyde were reported as 0.00, 0.03, 0.19, and 0.33 respectively). However, satisfactory R_f values can be obtained on formamide-treated paper for a number of indole acids and aldehydes using either a formamide-saturated benzene – ethyl acetate (3:1) mixture or dichloromethane (almost saturated with formamide) as the running solvents. The results obtained with 12 indole compounds are given in Table I. One advantage offered by this type of procedure over the

TABLE I

R_f values of some indole derivatives obtained using circular chromatography on formamide-treated paper*

	Compound	Average R_f† value in solvent S_1	Average R_f† value in solvent S_2	Source of compound
A	2-Indolecarboxylic acid	39	28	Prepared by the method of Kermack *et al.* (9)
B	3-Indolecarboxylic acid	37	22	Prepared by the method of Doyle *et al.* (8)
C	3-Indoleacetic acid	37	36	Eastman Kodak Company
D	3-Indole-β-propionic acid	56	54	Regis Chemical Company
E	3-Indole-γ-butyric acid	70	69	Regis Chemical Company
F	5-Hydroxy-3-indoleacetic acid	05	05	Regis Chemical Company
G	3-Indoleacrylic acid	44	35	Sigma Chemical Company
H	3-Indolelactic acid	04	04	Sigma Chemical Company
I	3-Indoleglyoxylic acid	04	04	‡
J	3-Indolecarboxaldehyde	62	72	Regis Chemical Company
K	3-Indoleacetaldehyde	88	94	‡
L	3-Indoleacetonitrile	91	95	Regis Chemical Company

* = The chromatography was carried out at room temperature.
† = (R_f values × 100).
‡ = Samples supplied through the courtesy of Dr. F. Wightman and Mr. M. Chisholm of the National Research Council (Prairie Regional Laboratory).
S_1 = benzene/ethyl acetate (3:1), saturated with formamide.
S_2 = dichloromethane containing 1.4% formamide by volume.

more conventional systems (e.g. isopropanol/aqueous ammonia) is the speed with which the separations can be achieved. The current investigation was carried out using the circular method exclusively and only ca. 35 minutes was required to complete runs as opposed to the 8 to 12 hours required with many other systems.

These investigations, which were supported by grants from the Saskatchewan Government, the Department of National Health and Welfare (Ottawa), and the Rockefeller Foundation, are continuing and further results will be reported in due course.

Experimental

Indoles

The sources of the indole derivatives used in this investigation are given in Table I.

Paper Chromatographic Procedure

(i) Apparatus

A modified Kawerau* Circular Chromatography apparatus (cf. 2, p. 40) was employed. The glass capillary solvent feed supplied with the apparatus was found to be unsatisfactory with the solvent systems used and was replaced by a wick of $\frac{1}{8}$-in. soft cotton yarn; the solvent was contained in a 6-cm petri dish.

(ii) Paper Disks

Paper disks (diameter = 26 cm) of Whatman 3MM paper were dipped into a 30% solution of formamide (Reagent grade; pH = 7)† in acetone, blotted free

*As supplied by the Shandon Scientific Co.
†The pH of the formamide was adjusted to 7 by the careful addition of the appropriate amount of either "880" ammonia or 98% formic acid.

of excess reagent, and allowed to dry in air for approximately 20 minutes prior to use.

(iii) Solvents

(a) S_1.—A mixture of benzene (dried over sodium; 3 parts) and ethyl acetate (Reagent grade; 1 part) was saturated with formamide by shaking with a slight excess of formamide, allowing the mixture to stand, and using the clear supernatant layer.

(b) S_2.—Dichloromethane (spectro grade) containing 1.4% formamide by volume. (This is slightly less than the amount required to saturate the solvent, but in view of the high volatility of dichloromethane it was found that with higher formamide concentrations the mixture often became cloudy, due to the separation of free formamide, and gave unsatisfactory results. Gröger has also pointed out that when using the formamide–dichloromethane system the mobile phase must not be allowed to become cloudy (10).)

(iv) Detection

Substances A, B, C, D, E, F, G, H, and L give positive reactions (red to violet colors) with Ehrlich's reagent. (Heating was necessary to obtain a color with compound A.) Substances I, J, and K gave orange or brown colors with 2,4-dinitrophenylhydrazine.

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THE RATE OF DESTRUCTION OF CIRCULATING ADRENALINE IN THE RABBIT EAR, BY A RECIRCULATION METHOD

R. H. Stinson

The blood vessels of the rabbit ear constrict strongly in the presence of adrenaline or noradrenaline. By perfusing an isolated rabbit ear at constant

flow and adding one of these pressor drugs to the perfusate we can measure the increase in the "driving pressure", at the arterial cannula, which is required to maintain a constant flow. This increase in pressure will be related to the concentration of the pressor drug. If the pressor drugs concerned are inactivated in the ear, then the effluent will have a lower concentration than the influent. If a recirculating system is set up so that the effluent is constantly pumped back into the ear, then the driving pressure will get less and less if some of the drug is destroyed each time it passes through the ear. Thus, the isolated rabbit ear could be used both to inactivate the catecholamines and to give a continuous assay of the quantity of drug present.

The method of isolation of the circulation of the ear is fairly standard and has been described in an earlier paper (1). The constant flow perfusion (2.4 ml/min) was obtained with a Sigmamotor pump. A pressure transducer (Statham) was attached to the arterial cannula and the driving pressure recorded. The perfusion fluid was a phosphate-buffered Ringer's solution (pH 7.4). Recirculation of perfusate for a few minutes before addition of the adrenaline permitted the perfusate to pick up enough blood to complex with the heavy metal ions unavoidably present in the perfusate and prevent in vitro oxidation of the adrenaline. Figure 1B illustrates the protection that can be obtained merely by recirculating the perfusate for a few minutes before adding the adrenaline. In both the recirculation (rc) and single passage (s.p.) portions of the recording the same concentration of adrenaline (3×10^{-8} g/ml) was employed. The protecting influence of blood can be seen in the recirculation portion. There also appears to be a slight potentiation of effect since exponential extrapolation (broken lines) back to the time of addition of adrenaline predicts a somewhat lower response for the single passage (i.e. blood-free) portion of the recording. This, however, may be an artefact since we do not know that the destruction is exponential from the start. Quantitative experiments indicated that addition of blood 0.5% by volume to the perfusate would always prevent in vitro oxidation.

Figure 1A shows the very slow destruction of adrenaline that occurs when it is protected by blood picked up in recirculation. At *a*, the adrenaline (3×10^{-8} g/ml) was added to 10 ml of recirculating Ringer's solution. The mean driving pressure increased 130 mm Hg, at *b*. Eighty minutes later, at *c*, the pressure was still 45 mm Hg above the original baseline. Since the relationship between concentration and increase in pressure is approximately linear in the range of concentrations concerned (2), this means that one third of the adrenaline was still present after 80 minutes. At *c*, the ear was washed out with fresh Ringer's solution and the driving pressure quickly returned to the original baseline. Then, at *d*, the same concentration of adrenaline was administered under the same conditions as at *a*, and the response was the same, showing that the decrease during 80 minutes was a real measure of a slow destruction of adrenaline and not fatigue. Similar results were obtained with six other ears.

The rate of disappearance in Fig. 1A is only 0.1 μg/kg/min. Lund (3) has measured the maximum rate of destruction of adrenaline in perfused rabbit

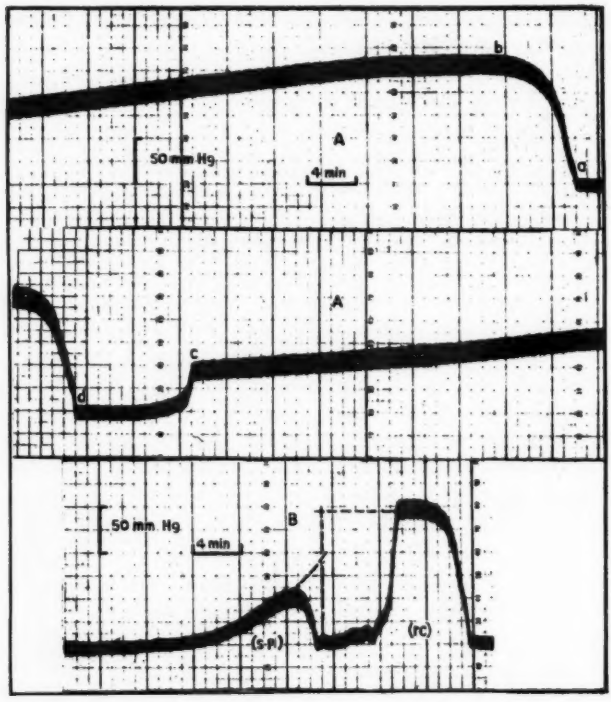

FIG. 1. (A) Pressure changes in response to recirculation of Ringer's solution containing adrenaline (3×10^{-8} g/ml).
(B) Contrast for the same concentration of adrenaline (3×10^{-8} g/ml) between recirculation (rc) and single passage (s.p.) of Ringer's solution.

liver as 10 mg/kg/min and in the perfused hind limb as 3 μg/kg/min in the presence of blood. It would seem then that the rate of destruction of humoral or exogenous adrenaline in such peripheral tissues as the rabbit ear is so slow as to be negligible compared with the rate in the liver, for example, and that the rapid disappearance of adrenaline effects in such tissues is not due to local destruction of the adrenaline, but rather due to the adrenaline being swept out by the blood stream and destroyed elsewhere. This would suggest, at least for the rabbit ear, that the well-known local control of acetylcholine by cholinesterase at cholinergic nerve endings either does not have an analog at the adrenergic endings or, if an analogous system exists, it cannot destroy appreciable amounts of circulating catecholamines.

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CONTENTS OF VOLUME 38

Acheson, R. M. and **Dearnaley, D. P.** Some constituents of the urine of normal and schizophrenic individuals. Part II, 503.
Adamkiewicz, V. W., Fitko, R. J., and **Fortier, A. A.** Hypoglycemic drugs and the dextran 'anaphylactoid' inflammation, 823.
Adie, P. A. See McPhail, M. K., 935, 945.
Akabane, Y. See Takahashi, Y., 1149.
Allen, C. E. See Dow, D. S., 1471.
Anastassiadis, P. A. Combined estimates in colorimetric determinations with high variability, 1223.
Anderson, D. G. and **McCalla, A. G.** Sedimentation of crystalline globulin from cucurbit seeds, 275.
Anderson, D. G., McCalla, A. G., and **McCalla, D. R.** Electrophoretic properties of crystalline globulin from cucurbit seeds, 657.
Aprile, M. A. See Moloney, P. J., 1216.
Arboit, T. See Isler, H., 703.
Armstrong, J. E. See Larsen, B. A., 813.
Axelrad, A. See Siminovitch, L., 425.
Baer, E., Buchnea, D., and **Gróf, T.** The chromatographic separation of homologous phosphatides, 853.
Baer, E. and **Gróf, T.** Synthesis of L-α-cephalins containing fatty acids of shorter chain length. Water-soluble glycerolphosphatides II, 859.
Baichwal, R. S., Baxter, R. M., Kandel, S. I., and **Walker, G. C.** Antifungal action of salicylanilide. II, 245.
Bain, G. O. *With the technical assistance of* **Van Kessel, M.** Agglutinin responses to *S. typhi* H antigen in intact and sublethally irradiated mice following the intraperitoneal injection of homologous spleen cell suspensions, 621.
Baker, D. G. Electrolyte metabolism in the rat exposed to a low environmental temperature. II, 205.
See also Clark, G. M., 320.
Bauld, W. S. See Goldzieher, J. W., 233.
Bauld, W. S., Givner, M. L., Engel, L. L., and **Goldzieher, J. W.** The fluorescence of eight natural estrogens in sulphuric acid, 213.
Baxter, R. M. See Baichwal, R. S., 245.
Beare, J. L. See Murray, T. K., 663.
Beare, J. L., Murray, T. K., and **Campbell, J. A.** Responses of two strains of rats to rapeseed oil and corn oil, 187.
Beaton, J. R. Metabolic effects of urea administration on acute hypothermia in rats, 709.
Beaton, J. R. and **Hunter, J.** Therapy of acute hypothermia with special reference to urea. A preliminary report, 305.
Beck, I. T., Pinter, E., McKenna, R. D., and **Griff, H.** The selective proteolytic enzyme inhibitory action of benzethonium chloride, 25.
Bélanger, L. F. See Bois, P., 585; Cipera, J. D., 807.
Beveridge, J. M. R. See Kuksis, A., 95; Valberg, L. S., 365.
Bidwell, R. G. S. See Larsen, B. A., 51.
Biely, J. See Wood, J. D., 19.
Birmingham, M. K., Kurlents, E., Lane, R., Muhlstock, B., and **Traikov, H.** Effects of calcium on the potassium and sodium content of rat adrenal glands, on the stimulation of steroid production by adenosine 3′,5′-monophosphate, and on the response of the adrenal to short contact with ACTH, 1077.
Bogoch, A. See Daniel, E. E., 777.
Bois, P., Byrne, E. H., and **Bélanger, L. F.** Effect of magnesium deficiency on the regeneration of mast cells after treatment with 48/80 in rats, 585.
Bone, D. H. and **Hochster, R. M.** An anomalous tricarboxylic acid cycle in *Acetobacter melanogenum*, 193.
Booyens, J. and **Hervey, G. R.** The pulse rate as a means of measuring metabolic rate in man, 1301.
Boyd, C. E., Boyd, E. M., and **Deyette, A. A.** Loss of weight and lipid shifts in organs of the digestive tract of tumor-bearing rats in relation to tumor size, 673.
Boyd, E. M. See Boyd, C. E., 673.
Branion, H. D. See Gray, J. A., 435.
Brown, S. A., Towers, G. H. N., and **Wright, D.** Biosynthesis of the coumarins. Tracer studies on coumarin formation in *Hierochloë odorata* and *Melilotus officinalis*, 143.
Buchnea, D. See Baer, E., 853.
Burr, L. H. and **McLennan, H.** The apparent extracellular space of mammalian skeletal muscle. A comparison of the inulin space in normal and dystrophic mouse tissues, 829.

Butt, W. D. and **Lees, H.** The biochemistry of the nitrifying organisms. Part 7. The phosphate compounds of *Nitrobacter* and the uptake of orthophosphate by the organism, 1295.
Byrne, E. H. See Bois, P., 585.
Calaresu, F. R. and **Jaques, L. B.** Thrombocytopenia in the experimental production of hemorrhagic death by multiple factors, 1275.
Campbell, J. A. See Beare, J. L., 187; Middleton, E. J., 865; Morrison, A. B., 467; Murray, T. K., 663; Stainer, D. W., 1219.
Campbell, J., Green, G. R., Schönbaum, E., and **Socol, H.** Effects of exposure to cold and of diet on coenzyme A levels in tissues, 175.
Campbell, J., Green, G. R., and **Socol, H.** Effects of exposure to cold on acetylation in the rat, 171.
Carroll, K. K. Effects of diet on incorporation of acetate-1-C^{14} into cholesterol by rat liver slices, 649.
Champagne, M. and **Smith, D. B.** Degradation by hydrazine of bovine plasma albumin and lysozyme, 715.
Chang, V. M. and **Idler, D. R.** Biochemical studies on sockeye salmon during spawning migration. XII. Liver glycogen, 553.
Chefurka, W. Studies on the inhibition of the mitochondrial ATP-ase by reduction of the respiratory chain, 1195.
Cipera, J. D., Migicovsky, B. B., and **Bélanger, L. F.** Composition of epiphyseal cartilage. I. Changes in hexosamine and acetone extractable contents of epiphyseal cartilage of rachitic chicks following administration of vitamin D_3, 807.
Clark, G. M. and **Baker, D. G.** Radiation, the cellular approach, 320.
Clarke, D. W. See Forbath, N., 13.
Cohen, J. J. See Hobkirk, R., 769.
Cole, E. R. See Dalby, A., 1029.
Cole, E. R., Dalby, A., and **Mertz, E. T.** Partial purification of bovine antiplasmin (antifibrinolysin), 1021.
Coleman, I. W., Little, P. E., and **Grant, G. A.** The induction of methaemoglobinemia as an adjunct to therapy for tabun poisoning, 667.
Oxime mixtures and atropine in the protection of mice and rats from sarin poisoning, 1035.
Colhoun, E. H. Acetylcholine in *Periplaneta americana* L. IV. The significance of esterase inhibition in intoxication, acetylcholine levels, and nervous conduction, 1363.
Collier, H. B. See Tsen, C. C., 957, 981.
Common, R. H. See MacRae, H. F., 523.
Constantopoulos, G. A simple automatic stimulation timer, 395.
Crevier, M. See Dowd, G. F., 989.
Dalby, A. See Cole, E. R., 1021.
Dalby, A., Cole, E. R., and **Mertz, E. T.** Studies on the purification of bovine plasminogen (profibrinolysin), 1029.
Dale, D. G. See MacRae, H. F., 523.
Dales, S. Effects of anaerobiosis on the rates of multiplication of mammalian cells cultured in vitro, 871.
Dandiya, P. C., Johnson, G., and **Sellers, E. A.** Influence of variation in environmental temperature on the acute toxicity of reserpine and chlorpromazine in mice, 591.
Daniel, E. E. *With the technical assistance of* **Renner, S. A.** The activation of various types of uterine muscle during stretch-induced contraction, 1327.
Daniel, E. E., Wachter, B. T., Honour, A. J., and **Bogoch, A.** The relationship between electrical and mechanical activity of the small intestine of dog and man, 777.
Darcel, C. le Q. *With the technical assistance of* **Klassen, H. C.** Paper electrophoresis of sera and plasma from chicks affected with erythroblastosis, 383.
Das, B. C. Effectiveness of yeast, vitamin B_{12}, and alternative treatments on survival of Indian carp during the early period of life, 453.
Davies, R. O., Marton, A. V., and **Kalow, W.** The action of normal and atypical cholinesterase of human serum upon a series of esters of choline, 545.
Dearnaley, D. P. See Acheson, R. M., 503.
Denyes, A. and **Horwood, R. H.** A comparison of free adrenal cortical steroids in the blood of a hibernating and non-hibernating mammal, 1479.
Depocas, F. *With the assistance of* **Secours, V.** The calorigenic response of cold-acclimated white rats to infused noradrenaline, 107.
Deyette, A. A. See Boyd, C. E., 673.
Dingle, J. R. and **Hines, J. A.** Proteins in fish muscle. 16. Adenosinetriphosphatase activity of cod myosin and actomyosin, 1437.
Dow, D. S. and **Allen, C. E.** Steady-state oxidation of glucose in the diabetic rat, 1471.
Dowd, G. F. and **Crevier, M.** A simple capacitor transducer for measuring blood pressure in small experimental animals, 989.

Dresel, P. E. *With the technical assistance of* **Negrych, S.** Blockade of some cardiac actions of adrenaline by dichloroisoproterenol, 375.
Dryden, E. E. See Manery, J. F., 1281.
Duffield, J. C. See Manery, J. F., 1281.
Du Ruisseau, J.-P. Acetylaspartic acid and ammonia poisoning, 763.
Du Ruisseau (Duruisseau), J.-P. See Mori, K., 919.
Elcoate, P. V. See Millar, M. J., 1457.
Engel, L. L. See Bauld, W. S., 213; Goldzieher, J. W., 233.
Fagerlund, U. H. M. and **Idler, D. R.** Marine sterols. VI. Sterol biosynthesis in molluscs and echinoderms, 997.
Fazekas, Á. Gy., Fazekas, I. Gy., and **Rengei, B.** Experimental examination of the mechanism of pyramidon effect. III. Effect of high pyramidon doses on the glutamic acid – oxaloacetic acid transaminase activity in serum, 899.
Fazekas, I. Gy. See Fazekas, Á. Gy., 899.
Ferguson, R. B. See White, F. D., 443.
Findlay, D. See Hurst, R. O., 347.
Fischer, M. I. See Millar, M. J., 1457.
Fitko, R. J. See Adamkiewicz, V. W., 823.
Forbath, N. and **Clarke, D. W.** Factors influencing the distribution of pentoses in the isolated rat diaphragm, 13.
Fortier, A. A. See Adamkiewicz, V. W., 823.
Foster, T. S. See Willmer, J. S., 1387, 1393, 1399.
Fox, S. M. See MacLeod, R. A., 693.
Fried, J. See Perlman, D., 393.
Genest, J. See Sándor, T., 739.
Giguère, R. See Lamarche, G., 1159.
Giuffre, N. See Jackson, P. W., 1377; Perlman, D., 393.
Givner, M. L. See Bauld, W. S., 213; Goldzieher, J. W., 233.
Godin, C. Amino acid – protein interactions, 805.
Goldzieher, J. W. See Bauld, W. S., 213.
Goldzieher, J. W., Bauld, W. S., Engel, L. L., and **Givner, M. L.** Recording spectrofluorometers: Two new designs and their evaluation, 233.
Gordon, E. R. and **Kalant, H.** Modifications of the assay for small amounts of antidiuretic hormone, 1380.
Gorin, P. A. J. See Spencer, J. F. T., 157.
Gorin, P. A. J., Haskins, R. H., and **Spencer, J. F. T.** Biochemistry of the Ustilaginales. XIII. Observations on the structure and biosynthesis of 4-O-β-D-mannopyranosyl-D-erythritol, 165.
Biochemistry of the Ustilaginales. XIV. Bioassimilation of L-erythrulose into 4-O-β-D-mannopyranosyl-D-erythritol, 953.
Gornall, A. G., Grundy, H. M., and **Koladich, C. J.** Studies on aldosterone. III. Chronic effect on the blood pressure of rats, 43.
Gowdey, C. W. Cardiac output in acute experimental methemoglobinemia, 1411.
Grant, G. A. See Coleman, I. W., 667, 1035.
Gray, J. A., Olsen, E. M., Hill, D. C., and **Branion, H. D.** Effect of a dietary lysine deficiency on the concentration of amino acids in the deproteinized blood plasma of chicks, 435.
Green, G. R. See Campbell, J., 171, 175.
Griff, H. See Beck, I. T., 25.
Gróf, T. See Baer, E., 853, 859.
Grundy, H. M. See Gornall, A. G., 43.
Gyenes, L. and **Sehon, A. H.** Preparation and evaluation of polystyrene–antigen conjugates for the isolation of antibodies, 1235.
The use of polystyrene–allergen conjugates for the removal of antibodies from sera of allergic individuals, 1249.
Habeeb, A. F. S. A. The sulphydryl content of β-lactoglobulin, 269.
A new reagent for the guanidination of proteins, 493.
Habeeb, A. F. S. A. and **Smith, D. B.** The amino acid contents of horse globin and of its component polypeptides, 263.
Harpur, R. P. and **Waters, W. R.** Production of carbon dioxide and volatile acids by muscle from *Ascaris lumbricoides*, 1009.
Haskins, R. H. See Gorin, P. A. J., 165, 953.
Hawkins, K. See Hosein, E. A., 837.
Hawkins, W. W. See Larsen, B. A., 51, 813.
Heacock, R. A. and **Mahon, M. E.** A note on the paper chromatography of some indole acids and their derivatives, 1500.
Héon, M. See Lamarche, G., 1163.
Héroux, O. Mitotic rate in the epidermis of warm- and cold-acclimated rats, 135.

Héroux, O. and **Willmer, J.** Respiratory rate (Q_{O_2}) of isolated liver of white rats exposed to summer or winter outdoor environmental conditions, 1215.
Héroux, O. *With the technical assistance of* **Wright, D.** The effect of intermittent indoor cold exposure on white rats, 517.
Hervey, G. R. See Booyens, J., 1301.
Hill, D. C. See Gray, J. A., 435.
Hines, J. A. See Dingle, J. R., 1437.
Hobkirk, R. and **Cohen, J. J.** Some aspects of the enzymic hydrolysis of urinary 17-ketosteroid conjugates, 769.
Hochster, R. M. See Bone, D. H., 193.
Hochster, R. M. and **Nozzolillo, C. G.** Respiratory carriers and the nature of the reduced diphosphopyridine nucleotide oxidase system in *Xanthomonas phaseoli*, 79.
Honour, A. J. See Daniel, E. E., 777.
Hori, A. See MacLeod, R. A., 693.
Horwood, R. H. See Denyes, A., 1479.
Hosein, E. A. and **Smart, M.** The presence of anserine and carnosine in brain tissue, 569.
Hosein, E. A., Smart, M., and **Hawkins, K.** Aminoaciduria in rats after treatment with the ethyl ester of gamma-butyrobetaine, 837.
Hudson, A. J. and **Kini, M. M.** The preparation and some metabolic properties of rat spinal cord slices, 965.
Hull, A. R. See White, F. D., 443.
Hunter, J. See Beaton, J. R., 305.
Hurst, R. O. and **Findlay, D.** The action of pancreatic deoxyribonuclease on oligonucleotides from calf thymus deoxyribonucleic acid, 347.
Ibrahim, R. K. and **Towers, G. H. N.** Studies of hydrangenol in *Hydrangea macrophylla* Ser. I. Isolation, identification, and biosynthesis from C^{14}-labelled compounds, 627.
Idler, D. R. See Chang, V. M., 553; Fagerlund, U. H. M., 997; Reiner, E., 1499; Tsuyuki, H., 1173, 1177.
Idler, D. R., Schmidt, P. J., and **Ronald, A. P.** Isolation and identification of 11-ketotestosterone in salmon plasma, 1053.
Isler, H., Nadler, N. J., Arboit, T., Johnston, N., Peter, W., and **Tucker, E.** Thyroid enlargement produced by prolonged administration of iodine in the mouse, 703.
Israels, L. G. See Zipursky, A., 727.
Jackson, P. W. See Perlman, D., 393.
Jackson, P. W., Giuffre, N., and **Perlman, D.** Growth of Ehrlich ascites tumor cells in tissue culture, 1377.
Jaques, L. B. See Calaresu, F. R., 1275.
Johnson, G. See Dandiya, P. C., 591.
Johnston, N. See Isler, H., 703.
Kalant, H. See Gordon, E. R., 1380.
Kalow, W. See Davies, R. O., 545.
Kandel, S. I. See Baichwal, R. S., 245.
Kasting, R. and **McGinnis, A. J.** Use of glutamic acid-U-C^{14} to determine nutritionally essential amino acids for larvae of the blow fly, *Phormia regina*, 1229.
Kates, M. See Stanaćev, N. Ž., 297.
Kerr, A. See White, F. D., 443.
Kini, M. M. See Hudson, A. J., 965.
Klassen, H. C. See Darcel, C. le Q., 383.
Koladich, C. J. See Cornall, A. G., 43.
Krupka, R. M. and **Laidler, K. J.** A molecular mechanism for enzymatic dehydrogenations involving pyridine nucleotides, 1185.
Kuksis, A. and **Beveridge, J. M. R.** The paper chromatographic fractionation of plant steryl esters, 95.
Kurlents, E. See Birmingham, M. K., 1077.
Laham, S. Biological conversion of 4-nitrobiphenyl to an active carcinogen, 1383.
Laidler, K. J. See Krupka, R. M., 1185.
Lamarche, G., Giguère, R., and **Langlois, J. M.** Stimulation of the fasciculus subcallosus in the cat with implanted electrodes, 1159.
Lamarche, G., Langlois, J. M., and **Héon, M.** Unit study of the trigeminal projections in the reticular formation of the medulla oblongata in the cat, 1163.
Landaburu, R. H. See Seegers, W. H., 1405.
Landaburu, R. H. and **Seegers, W. H.** The acetylation of prothrombin, 613.
Lane, R. See Birmingham, M. K., 1077.
Langlois, J. M. See Lamarche, G., 1159, 1163.
Lanthier, A. See Sándor, T., 1167.
Larsen, B. A., Bidwell, R. G. S., and **Hawkins, W. W.** The effect of ingestion of disodium ethylenediaminetetraacetate absorption and metabolism of radioactive iron by the rat, 51.

Larsen, B. A., Hawkins, W. W., Leonard, V. G., and **Armstrong, J. E.** The effect of the prolonged intake of ethylenediaminetetraacetate on the utilization of calcium and iron by the rat, 813.
LaRue, T. See Zipursky, A., 727.
Lees, H. See Butt, W. D., 1295.
Leonard, V. G. See Larsen, B. A., 813.
LePage, G. A. See Paterson, A. R. P., 1129.
Levy, S. W. and **Vaillancourt, de G.** Effects of inflammation on proteins and enzymes in rat plasma, 575.
Lisk, R. D. A comparison of the effectiveness of intravenous, as opposed to subcutaneous, injection of progesterone for the induction of estrous behavior in the rat, 1381.
Little, P. E. See Coleman, I. W., 667, 1035.
Lucas, C. C. See Mookerjea, S., 757.
Lucis, O. J. and **Venning, E. H.** In vitro and in vivo effect of growth hormone on aldosterone secretion, 1069.
McCalla, A. G. See Anderson, D. G., 275, 657.
McCalla, D. R. See Anderson, D. G., 657.
McConnell, W. B. See Nath, R., 533, 903.
McConnell, W. B., Nath, R., and **Spencer, J. F. T.** Studies on wheat plants using carbon-14 compounds. XI. The relative incorporation of carbons 1, 2, and 3 of pyruvate, 33.
McGinnis, A. J. See Kasting, R., 1229.
McKenna, R. D. See Beck, E., 25.
McLaughlan, J. M. and **Morrison, A. B.** Evaluation of protein quality in mixed foods, 1378.
McLennan, H. See Burr, L. H., 829.
MacLeod, D. P. See Reynolds, A. K., 889.
MacLeod, R. A., Hori, A., and **Fox, S. M.** Nutrition and metabolism of marine bacteria. IX. Ion requirements for obtaining and stabilizing isocitric dehydrogenase from a marine bacterium, 693.
McMurray, W. C. The inhibition of oxidative and phosphorylative enzymes in rat liver mitochondria by aminoazobenzene derivatives, 1.
McPhail, M. K. and **Adie, P. A.** Penetration of radioactive isopropyl methylphosphonofluoridate (sarin) vapor through skin, 935.
The distribution of radioactive phosphorus in the blood and tissues of rabbits treated with tagged isopropyl methylphosphonofluoridate (sarin), 945.
MacRae, H. F., Dale, D. G., and **Common, R. H.** Formation in vivo of 16-epiestriol and 16-ketoestradiol-17β from estriol by the laying hen and occurrence of equol in hen's urine and feces, 523.
Madsen, N. B. The oxidation of succinate in extracts of *Xanthomonas phaseoli*, 481.
Mahon, M. E. See Heacock, R. A., 1500.
Manery, J. F., O'Niell, K. E., Meakin, J. W., Dryden, E. E., and **Duffield, J. C.** The effect of bound insulin on the respiratory rate and electrolytes of intact frog muscle, 1281.
Marko, A. M. and **Reynolds, F. B.** A colorimetric method for measuring indican, 253.
Martin, S. M. See Singh, K., 969.
Marton, A. V. See Davies, R. O., 545.
Mawson, C. A. See Millar, M. J., 1457.
Meakin, J. W. See Manery, J. F., 1281.
Mehrotra, K. N. Effect of an anticholinesterase on the cholinergic system in insect eggs, 1045.
Mertz, E. T. See Cole, E. R., 1021; Dalby, A., 1029.
Middleton, E. J., Morrison, A. B., and **Campbell, J. A.** Evaluation of protein in foods. VI. Further factors influencing the protein efficiency ratio of foods, 865.
Migicovsky, B. B. Inhibition of cholesterol biosynthesis by a mitochondrial extract, 339.
See also Cipera, J. D., 807.
Millar, M. J., Elcoate, P. V., Fischer, M. I., and **Mawson, C. A.** Effect of testosterone and gonadotrophin injections on the sex organ development of zinc-deficient male rats, 1457.
Milojevic, S. See Monkhouse, F. C., 475.
Moloney, P. J. and **Aprile, M. A.** Neutralization of cod insulin with antiserum: Precipitation of insulin–anti-insulin complex with ethanol–water, 1216.
Monkhouse, F. C. and **Milojevic, S.** Changes in thrombin generation and antithrombin titer following massive bleeding and transfusion in dogs, 475.
Mookerjea, S. and **Lucas, C. C.** Effect of lipotropic agents upon the net synthesis of cholesterol in the rat, 757.
Morgan, J. F. and **Morton, H. J.** Carbohydrate utilization by chick embryonic heart cultures, 69.
Morgan, J. F. and **Pasieka, A. E.** Amino acid metabolism of normal and malignant cell cultures, 399.

Morgan, J. F., Tolnai, S., and **Townsend, G. F.** Studies on the in vitro antitumor activity of fatty acids. II. Saturated dicarboxylic acids, 597.
Mori, K. and **Du Ruisseau (Duruisseau), J.-P.** Water and electrolyte changes in aging process with special reference to calcium and magnesium in cardiac muscle, 919.
Morrison, A. B. See McLaughlin, J. M., 1378; Middleton, E. J., 865.
Morrison, A. B. and **Campbell, J. A.** Studies on the nutritional value of defatted fish flour, 467.
Morton, H. J. See Morgan, J. F., 69.
Muhlstock, B. See Birmingham, M. K., 1077.
Murnaghan, M. F. Conduction block of terminal somatic motor fibers in tick paralysis, 287.
Murray, T. K. See Beare, J. L., 187; Stainer, D. W., 1219, 1467.
Murray, T. K., Beare, J. L., and **Campbell, J. A.** Effect of dietary oils on the depletion of vitamin A, 663.
Myers, D. K. Biochemical effects of irradiation on liver regeneration, 1059.
Irradiation of diphosphopyridine nucleotide in dilute aqueous solutions, 1255.
Catalase levels and radiosensitivity of liver cells, 1489.
Nadler, N. J. See Isler, H., 703.
Nath, R. See McConnell, W. B., 33.
Nath, R. and **McConnell, W. B.** Studies on wheat plants using carbon-14 compounds. XII. Utilization of serine-C^{14} with special reference to glycine labelling, 533.
Studies on wheat plants using carbon-14 compounds. XIII. Incorporation of α-amino adipic acid-6-C^{14} in wheat plants, 903.
Neelin, E. M. See Neelin, J. M., 355.
Neelin, J. M. and **Neelin, E. M.** Zone electrophoresis of calf thymus histone in starch gel, 355.
Neish, A. C. See Watkin, J. E., 559.
Newcombe, H. B. Genetic effects of ionizing radiation, 330.
Nichol, C. A. Metabolism of neoplastic cells and their response to certain drugs, 409.
Nowaczynski, W. J. See Sándor, T., 739.
Nozzolillo, C. G. See Hochster, R. M., 79.
Olsen, E. M. See Gray, J. A., 435.
O'Niell, K. E. See Manery, J. F., 1281.
Ozaki, K. and **Wetter, L. R.** Glyceric acid kinase isolated from a Polish variety of rapeseed (*Brassica campestris* L.), 125.
The distribution of acylases in the seeds of higher plants, 929.
Pasieka, A. E. Paper chromatography of complex biological materials by solvent redevelopment without prior removal of salts or proteins, 1137.
See also Morgan, J. F., 399.
Paterson, A. R. P. The development of resistance to 6-mercaptopurine in a subline of the Ehrlich ascites carcinoma, 1117.
Paterson, A. R. P. *With a note by* **LePage, G. A.** Thiopurine nucleotide synthesis in a 6-MP-resistant subline of the Ehrlich ascites carcinoma and growth inhibition by certain antimetabolites, 1129.
Perlman, D. See Jackson, P. W., 1377.
Perlman, D., Jackson, P. W., Giuffre, N., and **Fried, J.** Metabolism of progesterone and testosterone by mammalian cells growing in suspension culture, 393.
Peter, W. See Isler, H., 703.
Phillips, W. E. J. In vitro studies on the biosynthesis of ubiquinone, 1105.
Pinter, E. See Beck, I. T., 25.
Presutti, H. J. See Reynolds, A. K., 889.
Quastel, J. H. Introductory remarks. Symposium on some biochemical aspects of the cancer cell, 397.
See also Tenenhouse, A., 1311.
Reiner, E., Idler, D. R., and **Wood, J. D.** A hypocholesterolemic factor in marine sterols, 1499.
Rengei, B. See Fazekas, Á. Gy., 899.
Renner, S. A. See Daniel, E. E., 1327.
Reynolds, A. K., Presutti, H. J., and **MacLeod, D. P.** The pharmacology of the *Rauwolfia* alkaloids chandrine and serpakrine, 889.
Reynolds, F. B. See Marko, A. M., 253.
Riedel, B. E. See Rudzik, A. D., 845, 1003.
Robinson, G. A. A micromethod for the determination of sulphate by flame photometry, 643.
Ronald, A. P. See Idler, D. R., 1053.
Ronwin, E. The TAC method of tryptic enzyme activity determination, 57.
Rudzik, A. D. and **Riedel, B. E.** The effects of adrenalectomy and cortisone on zinc metabolism in the sex glands and adrenal of the male rat, 845.

The effects of hypophysectomy and ACTH on zinc metabolism in the sex glands and adrenals of the male rat, 1003.
Saffran, M. and **Sharman, D. F.** An eluter for paper chromatograms, 303.
Sándor, T. and **Lanthier, A.** Biosynthesis of two steroid glycols from 17α-hydroxyprogesterone by surviving human ovarian slices, 1167.
Sandor, T., Nowaczynski, W. J., and **Genest, J.** The metabolism of aldosterone by surviving dog and human liver slices, 739.
Sauer, F. Fatty acid, cholesterol, and acetoacetate biosynthesis in liver homogenates from normal and starved guinea pigs, 635.
Scaife, J. F. Protection of human red cell cholinesterase against inhibition by tabun and O,O-diethyl-S-2-diethylaminoethyl phosphorothiolate, 301.
Scaife, J. F. and **Shuster, J.** Tissue cholinesterase levels in sarin poisoning, 1087.
Schmidt, P. J. See Idler, D. R., 1053.
Schönbaum, E. See Campbell, J., 175.
Secours, V. See Depocas, F., 107.
Seegers, W. H. See Landaburu, R. H., 613.
Seegers, W. H. and **Landaburu, R. H.** Purification of prothrombin and thrombin by chromatography on cellulose, 1405.
Sehon, A. H. See Gyenes, L., 1235, 1249.
Sellers, E. A. Introduction. Symposium on the biological effects of radiation, 311.
Somatic effects of ionizing radiation, 326.
See also Dandiya, P. C., 591.
Sharman, D. F. See Saffran, M., 303.
Shuster, J. See Scaife, J. F., 1087.
Siminovitch, L. and **Axelrad, A.** Some biological problems in cancer biochemistry, 425.
Simpson, F. J. Preparation and properties of transketolase from pork liver, 115.
Singh, K. and **Martin, S. M.** Purification and properties of a protease from *Penicillium cyaneo-fulvum*, 969.
Smart, M. See Hosein, E. A., 569, 837.
Smith, D. B. See Champagne, M., 715; Habeeb, A. F. S. A., 263.
Socol, H. See Campbell, J., 171, 175.
Spence, W. P. See Vittorio, P. V., 819.
Spencer, J. F. T. See Gorin, P. A. J., 165, 953; McConnell, W. B., 33.
Spencer, J. F. T. and **Gorin, P. A. J.** The biosynthesis of erythritol and glycerol and *Torulopsis magnoliae*. Studies with C^{14}-labelled glucose, 157.
Stainer, D. W. and **Murray, T. K.** Isomerization of vitamin A by tissue homogenates, 1467.
Stainer, D. W., Murray, T. K., and **Campbell, J. A.** Isomerization of 11-*cis* vitamin A in vivo, 1219.
Stanaćev, N. Ž. and **Kates, M.** Synthesis of dipalmitoyl-L-α-glycerophosphoric acid derivatives, as possible intermediates for synthesis of glycerylphosphatides, 297.
Stegwee, D. The role of esterase inhibition in tetraethylpyrophosphate poisoning in the housefly, *Musca domestica* L., 1417.
Stephenson, N. R. The lack of effect of ascorbic acid on the thymolytic activity of hydrocortisone, 803.
Stinson, R. H. The rate of destruction of circulating adrenaline in the rabbit ear, by a recirculation method, 1502.
Strömblad, B. C. R. Lactic acid metabolism of the submaxillary gland of the cat, 1431.
Symposium on some biochemical aspects of the cancer cell, 397.
Symposium on the biological effects of radiation, 311.
Takahashi, Y. and **Akabane, Y.** Protein metabolism of rat brain slices, 1149.
Tappel, A. L. See Tsen, C. C., 539.
Tarr, H. L. A. The enzyme 5-phosphorylribose pyrophosphokinase in fish muscle, 683.
Tenenhouse, A. and **Quastel, J. H.** Amino acid accumulation in Ehrlich ascites carcinoma cells, 1311.
Tolnai, S. See Morgan, J. F., 597.
Tomlinson, N. and **Warren, R. A. J.** Lingcod muscle phosphomonoesterases. I. Acid phosphatases that hydrolyze *p*-nitrophenyl phosphate, 605.
Towers, G. H. N. See Brown, S. A., 143; Ibrahim, R. K., 627.
Townsend, G. F. See Morgan, J. F., 597.
Townsley, P. M. The in vitro incorporation of phosphorus-P^{32} into tobacco mosaic virus, 459.
Traikov, H. See Birmingham, M. K., 1077.
Triantaphyllopoulos, D. C. Effects of intravenous injections of the anticoagulant fraction of incubated fibrinogen on blood coagulation, 909.
Tsen, C. C. and **Collier, H. B.** The protective action of tocopherol against hemolysis of rat erythrocytes by dialuric acid, 957.

The relationship between the glutathione content of rat erythrocytes and their hemolysis by various agents in vitro, 981.

Tsen, C. C. and **Tappel, A. L.** Oxidation of sulphydryl groups in denatured egg albumin, 539.

Tsuyuki, H. and **Idler, D. R.** The metabolism of inositol in salmon. I. The distribution of radioactivity in acid-soluble extracts of coho salmon following intraperitoneal and intra-arterial injections of 2-C^{14}-myoinositol, 1173.

The metabolism of inositol in salmon. II. The role of nucleotides in relation to the metabolism of 2-C^{14}-myoinositol in coho liver, 1177.

Tucker, E. See Isler, H., 703.

Vaillancourt, de G. See Levy, S. W., 575.

Valberg, L. S. and **Beveridge, J. M. R.** Amino acid pattern in the blood of rats with dietary hepatic necrosis, 365.

Van Kessel, M. See Bain, G. O., 621.

Venning, E. H. See Lucis, O. J., 1069.

Vittorio, P. V. and **Spence, W. P.** The effect of 2-aminoethylisothiouronium (AET) on the respiration of mouse liver and spleen slices, 819.

Wachter, B. T. See Daniel, E. E., 777.

Walker, G. C. See Baichwal, R. S., 245.

Warren, R. A. J. See Tomlinson, N., 605.

Waters, W. R. See Harpur, R. P., 1009.

Watkin, J. E. and **Neish, A. C.** Biosynthesis of quercetin in buckwheat. Part III, 559.

Wetter, L. R. See Ozaki, K., 125, 929.

White, F. D., Kerr, A., Ferguson, R. B., and **Hull, A. R.** The effect of various denaturants on the haemoglobins of adult and cord blood. II. Urea as a denaturant, 443.

Whitmore, G. F. Radiation, its primary interactions, 312.

Willmer, J. See Héroux, O., 1215.

Willmer, J. S. Changes in hepatic enzyme levels after adrenalectomy. I. Phosphorylase, phosphoglucomutase, and phosphoglucoseisomerase, 1095.

The influence of adrenalectomy upon the activity of the hexosemonophosphate shunt in the livers and mammary glands of lactating rats, 1265.

Changes in hepatic enzyme levels after adrenalectomy. II. Glucose-6-phosphatase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase, 1449.

Willmer, J. S. and **Foster, T. S.** The influence of adrenalectomy and individual steroid hormones upon the metabolism of acetate-1-C^{14} by rat liver slices. I. Incorporation into proteins, 1387.

The influence of adrenalectomy and individual steroid hormones upon the metabolism of acetate-1-C^{14} by rat liver slices. II. Incorporation into cholesterol, 1393.

The influence of adrenalectomy and individual steroid hormones upon the metabolism of acetate-1-C^{14} by rat liver slices. III. Respiratory effects, 1399.

Wood, J. D. Dietary marine fish oils and cholesterol metabolism. 2. The effect of vitamin A and lingcod liver oil components on the serum cholesterol levels in chicks, 879.

See also Reiner, E., 1499.

Wood, J. D. and **Biely, J.** The effect of dietary marine fish oils on the serum cholesterol levels in hypercholesterolemic chickens, 19.

Wright, D. See Brown, S. A., 143; Héroux, O., 517.

Zipursky, A., LaRue, T., and **Israels, L. G.** The in vitro metabolism of erythrocytes from newborn infants, 727.

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CONTENTS

Page

Corrections i

Contents of Volume 38 1505

BIOCHEMISTRY

J. S. Willmer and T. S. Foster—The influence of adrenalectomy and individual steroid hormones upon the metabolism of acetate-1-C^{14} by rat liver slices. I. Incorporation into proteins 1387

J. S. Willmer and T. S. Foster—The influence of adrenalectomy and individual steroid hormones upon the metabolism of acetate-1-C^{14} by rat liver slices. II. Incorporation into cholesterol 1393

J. S. Willmer and T. S. Foster—The influence of adrenalectomy and individual steroid hormones upon the metabolism of acetate-1-C^{14} by rat liver slices. III. Respiratory effects 1399

Walter H. Seegers and Ricardo H. Landaburu—Purification of prothrombin and thrombin by chromatography on cellulose 1405

D. Stegwee—The role of esterase inhibition in tetraethylpyrophosphate poisoning in the housefly, *Musca domestica* L. 1417

J. R. Dingle and J. A. Hines—Proteins in fish muscle. 16. Adenosinetriphosphatase activity of cod myosin and actomyosin 1437

J. S. Willmer—Changes in hepatic enzyme levels after adrenalectomy. II. Glucose-6-phosphatase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase 1449

D. W. Stainer and T. K. Murray—Isomerization of vitamin A by tissue homogenates 1467

Dorothy S. Dow and C. E. Allen—Steady-state oxidation of glucose in the diabetic rat 1471

D. K. Myers—Catalase levels and radiosensitivity of liver cells 1489

Notes:

E. Reiner, D. R. Idler, and J. D. Wood—A hypocholesterolemic factor in marine sterols 1499

R. A. Heacock and M. E. Mahon—A note on the paper chromatography of some indole acids and their derivatives 1500

PHYSIOLOGY

C. W. Gowdey—Cardiac output in acute experimental methemoglobinemia . 1411

B. C. R. Strömblad—Lactic acid metabolism of the submaxillary gland of the cat 1431

M. J. Millar, P. V. Elcoate, M. I. Fischer, and C. A. Mawson—Effect of testosterone and gonadotrophin injections on the sex organ development of zinc-deficient male rats 1457

Arliss Denyes and Robert H. Horwood—A comparison of free adrenal cortical steroids in the blood of a hibernating and non-hibernating mammal . . . 1479

Notes:

R. H. Stinson—The rate of destruction of circulating adrenaline in the rabbit ear, by a recirculation method 1502